THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XXXIV SEPTEMBER-OCTOBER, 1958 NUMBER 5

HYPERTENSIVE VASCULAR DISEASE AS A CONSEQUENCE OF INCREASED ARTERIAL PRESSURE

QUANTITATIVE STUDY IN RATS WITH HYDRALAZINE-TREATED RENAL HYPERTENSION *

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Debate concerning the association between arterial hypertension and hypertensive vascular disease began 80 years ago in England with the formulation of contrasting concepts by Gull and Sutton[1] and by Mahomed.[2] It has continued since, largely at the clinico-pathologic level, and has not been resolved by some 25 years study of experimental hypertension. However, the experiments of Wilson and Byrom,[3] Byrom and Dodson,[4] and of Ledingham and Floyer[5] would seem to prove that relative normotension or hypotension, such as occurs also in the "endocrine kidney,"[6] protects the renal vascular bed of the rat from nephrosclerotic lesions. Since nephrosclerosis develops in kidneys exposed to high arterial pressure, it would seem to follow that hypertension is the primary cause of the vascular lesions. Unfortunately, the nature of these experiments is such that the differences observed are qualitative only.

There have been relatively few attempts, and these in small series,[7] to establish a quantitative association between the severity of hypertensive vascular disease and the concurrent level of arterial pressure. Nevertheless, many who are not fully convinced of this association believe that other factors, such as the presence[8] or absence[9] of intact kidney tissue, partly or wholly determine the occurrence of hypertensive vascular disease, especially of that which is acute, fulminant, and severely destructive.

* This investigation was supported in part by a grant (H-96) from the National Heart Institute, National Institutes of Health, Bethesda, Md., and a grant from the Cleveland Area Heart Society. A partial report was presented at the Society for the Study of Arteriosclerosis, Chicago, November, 1957.

Received for publication, February 7, 1958.

The debate has since been reopened by Koletsky[10] who found vascular lesions in rats made hypertensive by renal infarction, some of which were given a sympatholytic, presumably antihypertensive drug, Dibenamine. The problem is of considerable importance, since the current wide use of antihypertensive drugs is based on the premise that there is a direct association between high arterial pressure and hypertensive vascular disease. Indeed, such drugs have been shown to cause remission of the clinical syndrome of malignant hypertension[11] as well as healing of the associated vascular lesion.[12]

The present experiments were designed to resolve the issue experimentally by a quantitative study of the effects produced on hypertensive vascular disease by the prevention and remission of hypertension caused by renal infarction in rats.

Methods

The experiments were carried out in male Sprague-Dawley rats weighing 140 to 190 gm. and maintained on tap water and Purina Fox Chow. Renal hypertension was induced according to the method described by Loomis[13]: Under ether anesthesia the left kidney was exposed, the posterior branch of the renal artery was separated from the renal vein and ligated with silk; a right nephrectomy was performed in the same stage. The procedure causes a progressive rise in blood pressure; in some animals to levels of about 220 mm. of Hg over the course of 5 to 7 days. The rats uniformly develop severe and acute vascular lesions. In spite of a high degree of inbreeding in our strain of rats, and the use of a standard operative procedure, the severity of hypertension was individually unpredictable. This variability is probably due to individual variations in the distribution of branches of the renal artery.

Unilateral nephrectomy was performed in a group of animals, and the contralateral kidney was exposed and handled, but no arterial ligation was carried out; these sham-operated rats served as controls.

Since the aim of the experiments was to establish the quantitative association between hypertension and vascular disease, it was essential that the procedure used in preventing the onset of, or treating, established hypertension insure maintenance of arterial pressure at relatively stable levels during the whole treatment period. It was also necessary that the efficacy of treatment be verified by daily or twice daily observations of blood pressure. Hydralazine* was selected as a recognized, potent antihypertensive agent, known to be effective in

* Hydralazine (Apresoline, "Ciba") was kindly furnished by Dr. R. Gaunt, Ciba Pharmaceutical Products, Inc., Summit, N.J.

rats[14] and in man. Preliminary experiments were done to determine a mode of administration which would insure a lasting, predictable, antihypertensive effect.

To this end, 24 rats were placed according to weight into 4 groups of 6 animals each and subjected to the Loomis operative procedure. Group 1 was kept as control, while hydralazine was administered to the others by gavage once (group 2) or twice (group 3) daily, or by adding 40 mg. to each liter of drinking water (group 4). On the morning of the seventh day, 10 mg. per kg. of hydralazine was given by gavage to all rats except the controls; blood pressure was determined before and 2 hours after administration of this test dose. The results, summarized in Table I, indicate that intragastric administration once or twice daily did not control hypertension throughout the whole day, although most animals showed satisfactory antihypertensive responses 2 hours after gavage. In contrast, administration of hydralazine in drinking water did prevent the expected rise in blood pressure and was evidently fully effective throughout the day, since the seventh day test dose had little or no significant additional depressor effect. Having thus established an effective method of controlling hypertension, definitive experiments were aimed at prevention and remission of acute (series I) and chronic (series II) hypertensive vascular disease due to renal infarction.

TABLE I

Duration of Action of Hydralazine in Rats with Renal Infarction

Group	Treatment	Blood pressure in mm. of Hg		
			7 days	
		0 days	A*	P*
1	Control	129 (100–140)	174 (155–220)	183 (165–220)
2	Hydralazine gavage 10 mg. per kg. 1 ×/day	128 (115–140)	153 (115–200)	111 (90–170)
3	Hydralazine gavage 10 mg. per kg. 2 ×/day	128 (119–140)	152 (140–170)	117 (90–135)
4	Hydralazine, 40 mg. per l. in drinking water	121 (100–140)	135 (125–145)	123 (100–135)

* Blood pressure values before (A) and 2 hours after (P) administration of hydralazine by gavage (10 mg. per kg. of body weight) except in group 1 where blood pressure determinations were made at comparable intervals.

The experiments of brief duration (series I) were carried on for 7 and 15 days; 5 groups of animals were used. Details on grouping, treatment and number of animals are presented in Table II. The more

prolonged experiments of series II lasted 1 and 2 months and consisted of 6 groups; procedures are summarized in Table III. Blood pressure was determined by tail sphygmography.[15] At the end of each experiment, the animals were necropsied. Heart and kidney were removed, fixed in SUSA fluid, weighed, sectioned and stained with hematoxylin and eosin, periodic acid-fuchsin (PAS), and phosphotungstic acid hematoxylin (PTAH). These two tissues and also specimens of mesentery and pancreas were examined for the presence of vascular alterations.

TABLE II

Effects of Hydralazine During Acute Renal Hypertension (Series I)

Group	No. of rats	Treatment	Duration of experiment (days)	Percentage with extra-renal vascular disease	Heart wt. as percentage of body wt.
1	14	0	7	66%	.34% (.29–.41)
2	22	+	7	18	.32 (.28–.38)
3	22	0	15	68	.30 (.25–.39)
4	21	+*	15	57	.34 (.28–.40)
5	10	0†	7	0	.26 (.23–.28)

* Hydralazine started on seventh day.
† Sham-operated control.

TABLE III

Effects of Hydralazine During Chronic Renal Hypertension (Series II)

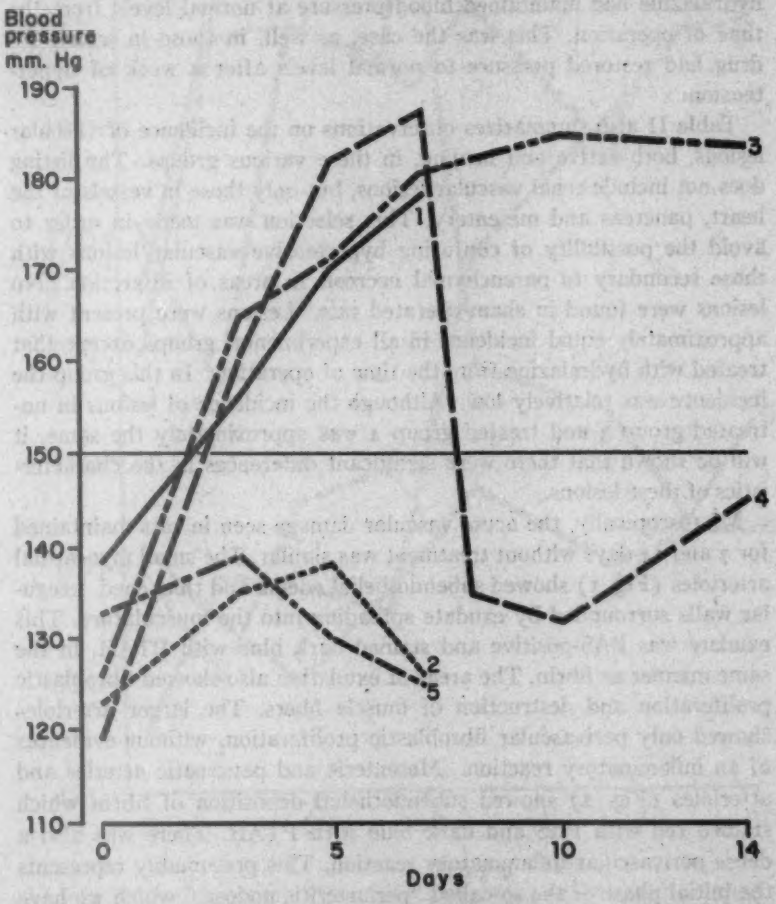
Group	No. of rats	Treatment	Duration of experiment (mos.)	Mortality	Percentage with extra-renal vascular disease	Heart wt. as percentage of body wt.	Gross arteritis
1	10	0	1	4	100%	.43% (.32–.64)	+
2	10	+	1	1	22	.32 (.23–.43)	0
3	10	0	2	2	85	.36 (.27–.49)	+
4	8	+	2	1	20	.37 (.33–.43)	0
5	10	+*	2	2†	70	.35 (.28–.46)	+
6	10	+‡	2	3†	90	.34 (.31–.41)	+

* Treatment was terminated on the 30th day and animals sacrified one month later.
† Mortality occurred after operation (group 5) or before initiation (group 6) of treatment.
‡ Treatment was initiated on the 30th day and animals sacrificed one month later.

Results

Series I. Experiments of Brief Duration

The means of consecutive blood pressure readings in the various groups are indicated in Text-figure 1. Taking 150 mm. of Hg as the upper limit of normal pressure, it can be seen that the means of the pressures in rats with renal infarction exceeded this level about the third day and increased further to the seventh day, stabilizing at about 180 mm. of Hg in animals observed to the 14th day. Sham-operated rats showed no significant changes in blood pressure. The means of blood pressure in rats with renal infarction which had received hy-



Text-figure 1. Blood pressure changes in animals of Series I (experiments of brief duration).

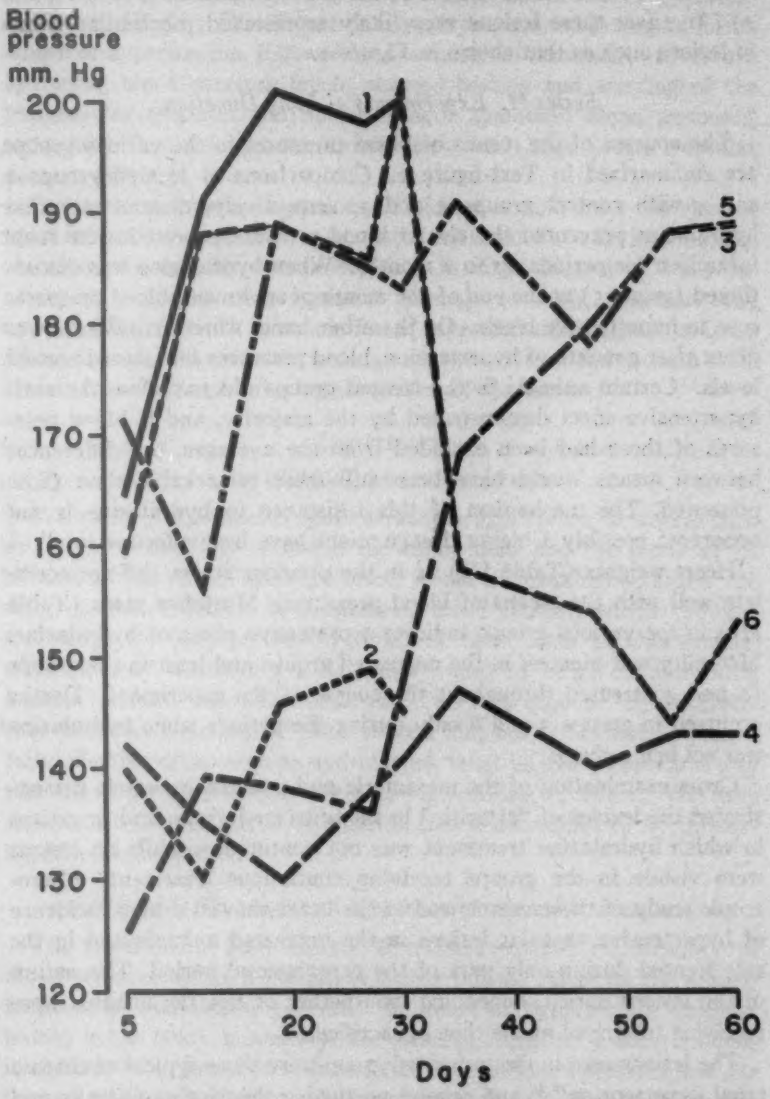
dralazine from the time of operation were nearly the same as those observed in the sham-operated group. Rats given hydralazine from the seventh day showed a prompt decrease of blood pressure to normal level, with a tendency to increase slightly in succeeding days, although hypertensive levels were not attained.

The means and ranges of heart weights per 100 gm. of body weight are listed in Table II. This datum is usually a function of blood pressure, and, accordingly, heart weight was increased in untreated rats with renal infarction as compared with the sham-operated controls. However, there was some disparity in this association, since heart weight was also increased in rats in which the administration of hydralazine had maintained blood pressure at normal levels from the time of operation. This was the case, as well, in those in which the drug had restored pressure to normal levels after a week of hypertension.

Table II also summarizes observations on the incidence of vascular lesions, both active and healing, in these various groups. The listing does not include renal vascular lesions, but only those in vessels of the heart, pancreas and mesentery. This selection was made in order to avoid the possibility of confusing hypertensive vascular lesions with those secondary to parenchymal necrosis in areas of infarction. No lesions were found in sham-operated rats. Lesions were present with approximately equal incidence in all experimental groups except that treated with hydralazine from the time of operation. In this group the incidence was relatively low. Although the incidence of lesions in untreated group 3 and treated group 4 was approximately the same, it will be shown that there were significant differences in the characteristics of these lesions.

Microscopically, the acute vascular damage seen in rats maintained for 7 and 14 days without treatment was similar. The small myocardial arterioles (Fig. 1) showed subendothelial edema and thickened, irregular walls surrounded by exudate spreading into the musculature. This exudate was PAS-positive and stained dark blue with PTAH, in the same manner as fibrin. The areas of exudation also showed fibroblastic proliferation and destruction of muscle fibers. The larger arterioles showed only perivascular fibroblastic proliferation, without evidences of an inflammatory reaction. Mesenteric and pancreatic arteries and arterioles (Fig. 2) showed subendothelial deposition of fibrin which stained red with PAS and dark blue with PTAH. There was also a dense perivascular inflammatory reaction. This presumably represents the initial phase of the so-called "periarteritis nodosa," which we have considered an expression of hypertensive vascular disease in the rat.[16]

In rats treated with hydralazine from the time of operation, the few lesions seen were similar in their general characteristics but less intense than those just described. In rats treated with hydralazine from the seventh to the 15th day (group 4), lesions were very different from



Text-figure 2. Blood pressure changes in animals of Series II (experiments of long duration).

those in the corresponding untreated animals (group 3). The differences consisted (a) in the disappearance of the characteristics of acute and progressive lesions, and (b) in their replacement by lesions which were predominantly fibrous. The latter exhibited diffusion of the fibrinoid exudate and disappearance of the cellular infiltration (Figs. 3 and 4). In effect these lesions very likely represented the healing phase of lesions such as that shown in Figure 2.

Series II. Experiments of Long Duration

The courses of the means of blood pressures in the various groups are summarized in Text-figure 2. Comparisons of treated groups 2 and 4 with control groups 1 and 3, respectively, demonstrate that hydralazine prevented the rise of blood pressure expected after renal infarction for periods up to 2 months. When hydralazine was discontinued (group 5) at the end of one month of treatment, blood pressures rose to hypertensive levels. On the other hand, when hydralazine was given after a month of hypertension, blood pressures fell toward normal levels. Certain animals in the treated groups did not show the antihypertensive effect demonstrated by the majority, and if blood pressures of these had been excluded from the averages, the differences between means would have been still more remarkable than those presented. The mechanism of this resistance to hydralazine is not apparent; possibly a higher dosage might have been effective in all.

Heart weights (Table III) as in the previous series, did not correlate well with the means of blood pressures. Mortality rates (Table III) in the various groups indicate a protective effect of hydralazine. Mortality was greatest in the untreated groups and least in the groups (2 and 4) treated throughout the course of the experiment. Deaths occurred in groups 5 and 6 only during the periods when hydralazine was not being given.

Gross examination of the mesenteric and pancreatic vessels demonstrated the lesions of "arteritis" in the untreated group and in groups in which hydralazine treatment was not continuous, while no lesions were visible in the groups receiving continuous treatment. Microscopic study of these vessels and of the heart showed a high incidence of hypertensive vascular lesions in the untreated animals and in the rats treated during only part of the experimental period. The nature of the lesions varied, depending on whether or not the animals were receiving treatment at the time of sacrifice.

The lesions seen in the untreated group were those typical of chronic renal hypertension[13,15] and require no further description (Figs. 5 and 6). Lesions were not entirely prevented by treatment, but were less

severe (Fig. 7) and less frequent in the treated groups. The appearance of lesions in treated animals may be ascribed in part to "escape" or resistance to treatment in individual rats. Animals treated for one month and surviving during a subsequent month of hypertension showed lesions corresponding to those seen in the untreated hypertensive group (Fig. 8). The lesions of animals maintained during an initial month of hypertension, followed by one month of treatment with return to normal blood pressure levels, showed healing and scarring of the perivascular exudate; the fibrous sheath contained blood pigments, suggesting that prior to treatment there had been severe vascular damage (Fig. 9).

Sections were made of portions of kidney remote from the sites of gross infarction. In 4 of 10 rats treated continuously with hydralazine, showing only mild hypertensive lesions in other sites, there were unexpected evidences of progressive damage to large arterioles and arteries (Fig. 10) with accumulation of subendothelial fibrinoid masses. These extended out into the vessel wall, provoking a fibroblastic reaction and causing a progressive narrowing of the lumen.

Discussion

Use of Antihypertensive Drugs

Preliminary experiments demonstrate the importance, in studies such as these, of carefully selecting the agent used and the mode of its administration. Since the experiment would be meaningless unless the drug was fully effective in maintaining blood pressure within the normal range for long periods of time, an agent such as Dibenamine, which is only transiently a depressor[17] and to which animals soon become tolerant, would be unsuitable. This may account for the difference between Koletsky's[10] observations and ours. Moreover, even a fully effective drug, such as hydralazine, must be given in such a way that blood pressure is controlled throughout the 24 hours. We have shown that the best mode of securing this is accomplished by dissolving it in drinking water.[7]

Effects of Treatment on Hypertensive Vascular Disease

The experiments have clearly demonstrated that maintenance of blood pressure within a normal or nearly normal range by the administration of hydralazine prevents the onset of hypertensive vascular lesions in the heart, mesentery, and pancreas. As noted, some animals in the treated group showed minor increases in blood pressure and the few mild lesions found in this group could be ascribed to an escape from the effect of treatment or to variations in individual susceptibility.

In any case, the averages of blood pressure and the data on lesions in the two groups continuously treated after renal infarction supported the thesis that the occurrence of lesions was in great part a function of increased blood pressure.

This proposition was further supported by the nature and extent of the lesions in animals treated for an initial period, during which blood pressure was maintained within normal levels and from whom treatment was subsequently withdrawn. This resulted in a prompt rise of blood pressure to hypertensive levels and the development of arterial lesions. The experiments emphasized the importance of continuous, effective maintenance of normal blood pressure in the prevention of vascular alteration. They also indicated that maintenance of blood pressure at normal levels for periods up to one month after renal infarction did not alter the functioning of the renal pressor mechanism. This was evidently continuously active and caused hypertension as soon as the drug was withdrawn.

In another study[12] we observed the lesions of healed malignant hypertension at necropsy in patients who had been under prolonged antihypertensive treatment for this condition. The experiments carried out in animals made hypertensive and subsequently treated with hydralazine paralleled this clinical phenomenon, and in a general way, the healing noted in the animals corresponded to that seen in human beings. In the experiments of long duration, healing of mesenteric lesions left a large residuum of abnormal vessels. However, the fact that the disease had been abated was shown in the disappearance of the inflammatory reaction from the scarred, fibrous vessels.

Nature of Hypertensive Vascular Disease in the Rat

Goldblatt[13] recently reaffirmed his contention that the mesenteric lesions produced in the rat by various procedures which increase blood pressure did not correspond to arteriolar sclerosis or necrosis as seen in human essential hypertension. This was partly because they occurred in larger vessels than arterioles and partly because he accepted them as lesions of periarteritis nodosa. The implication seemed to be that they were also nonspecific lesions, unrelated to hypertension. We would, of course, agree that these do not correspond directly to the arteriolar lesions of human hypertension. The present experiments have clearly shown that they are, as we have previously stated, not the equivalents of the lesions observed in clinical periarteritis nodosa but the species specific lesions of hypertensive vascular disease in the rat. They did not occur if the blood pressure was controlled; they did appear when blood pressure rose to hypertensive levels. Moreover, they

did heal when, after a phase of controlled hypertension, blood pressure was restored to or towards normal.

In the paper referred to,[12] attention was drawn to the fact that active, progressive lesions, particularly of the larger renal arteries were found in some patients whose antihypertensive treatment had seemed quite effective. A corresponding phenomenon affecting smaller renal arteries and arterioles seemed to occur in some rats with renal infarction under continuous and apparently effective antihypertensive treatment. These showed renal lesions but little or no hypertensive vascular disease in the extra-renal sites examined. The observations demonstrated that both in the rat and in man the renal vasculature was especially susceptible to hypertensive vascular lesions.

Heart Weight and Hypertension

Attention has been drawn to the failure of heart weight to show the expected association with blood pressure in animals under continuous treatment with hydralazine. Clinically, it is recognized that even prolonged and effective treatment of severe hypertension with hydralazine may fail to restore heart size to normal and that, in general, care should be exercised in the vigorous use of this drug in patients with severe hypertensive heart disease.[19,20] We have related this phenomenon to the tachycardia and increased cardiac output produced by hydralazine. The present experiments have indicated that in the rat, administration of hydralazine did not cause heart size to revert to normal in animals previously hypertensive. It actually caused increase of heart size in animals whose blood pressure had not been allowed to rise to hypertensive levels. Here, as in man with hyperthyroidism,[21] it is suggested that tachycardia and increased cardiac output cause cardiac hypertrophy in the rat.

Summary and Conclusions

The quantitative associations between levels of arterial blood pressure and the occurrence and character of hypertensive vascular lesions (myocardial, mesenteric, and pancreatic) were tested in rats subjected to partial renal infarction and uninephrectomy. The levels of blood pressure were controlled by the administration of hydralazine in drinking water.

This mode of administration of the drug was found to secure stable control of blood pressure at normal levels in most animals and, in the dose used, was apparently maximally effective.

In experiments of short duration (7 to 14 days), acute vascular lesions were found in the majority of animals with untreated hyperten-

sion. These were largely prevented by maintenance of blood pressure within a normal range and, when they had been allowed to develop, were found to lose their acute characteristics when remission of hypertension was induced by hydralazine. A similar sequence was established in experiments of longer duration (1 to 2 months). The latter experiments also showed that control of renal hypertension by a drug for one month did not prevent the onset of severe hypertension and vascular lesions when the drug was withdrawn. The data demonstrated a close association between blood pressure levels and the presence and character of the vascular lesions.

The observations confirm the view that mesenteric "arteritis" of rats with hypertension is in fact an expression of hypertensive vascular disease.

The sensitivity of the renal vascular bed to hypertension was demonstrated in a group of hypertensive, hydralazine-treated rats which showed slight extra-renal vascular alterations but, in some cases, severe and apparently progressive lesions in renal vessels.

In accordance with previous clinical observations, treatment of renal hypertension in rats did not restore heart size to normal. On the contrary, continuous treatment with hydralazine in doses sufficient to prevent the onset of hypertension was found to increase heart size, presumably as a result of increased heart rate and output.

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The authors are indebted to Miss Sheila Keefe, Mrs. E. Haskill, and Mr. A. Roth for excellent technical assistance.

[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Heart from rat with renal infarction, sacrificed on seventh day, showing acute lesions consisting of fibrinous deposits around vessel walls with infiltration into adjacent parenchyma. This is accompanied by marked cellular proliferation. Periodic acid-Schiff stain. × 220.

FIG. 2. Acute arterial lesion in the mesentery of rat on seventh day after renal infarction, showing subendothelial deposit of PAS-positive material. PAS stain. × 220.

FIG. 3. Small artery in the pancreas of rat treated with hydralazine from seventh to 15th day after renal infarction. The vessel shows increased thickness of its wall due to deposition of collagen. There is no cellular reaction. PAS stain. × 220.

FIG. 4. Small artery in heart, demonstrating similar lesion to that shown in Figure 3. PAS stain. × 220.

FIG. 5. Fully developed lesions of "periarteritis nodosa" in a pancreatic artery, showing subendothelial deposits of PAS-positive material. Rat surviving renal infarction; no treatment given. PAS stain. × 45.

FIG. 6. Same lesions as shown in Figure 5 but stained with phosphotungstic acid hematoxylin. Note the dark subendothelial deposit indicative of fibrin. × 50.

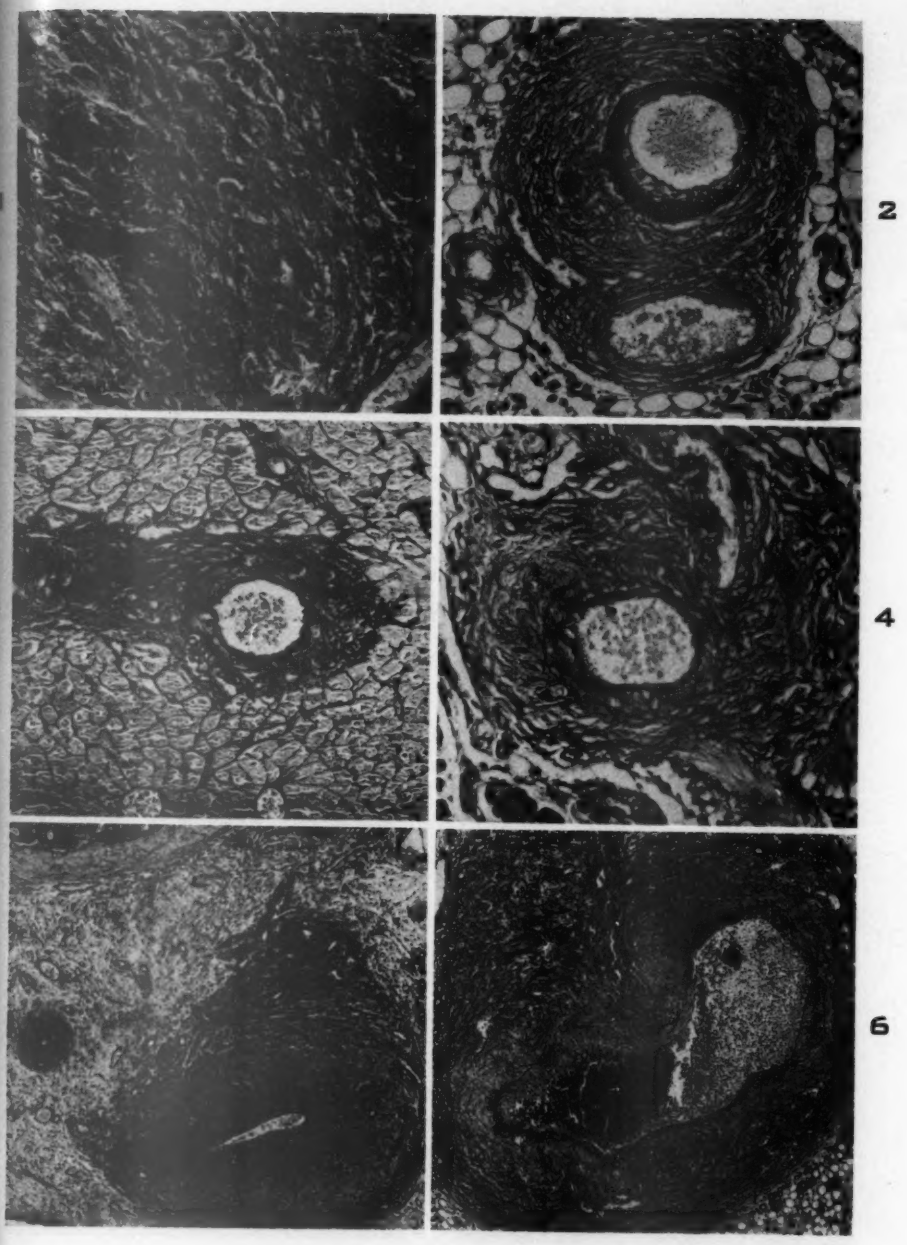
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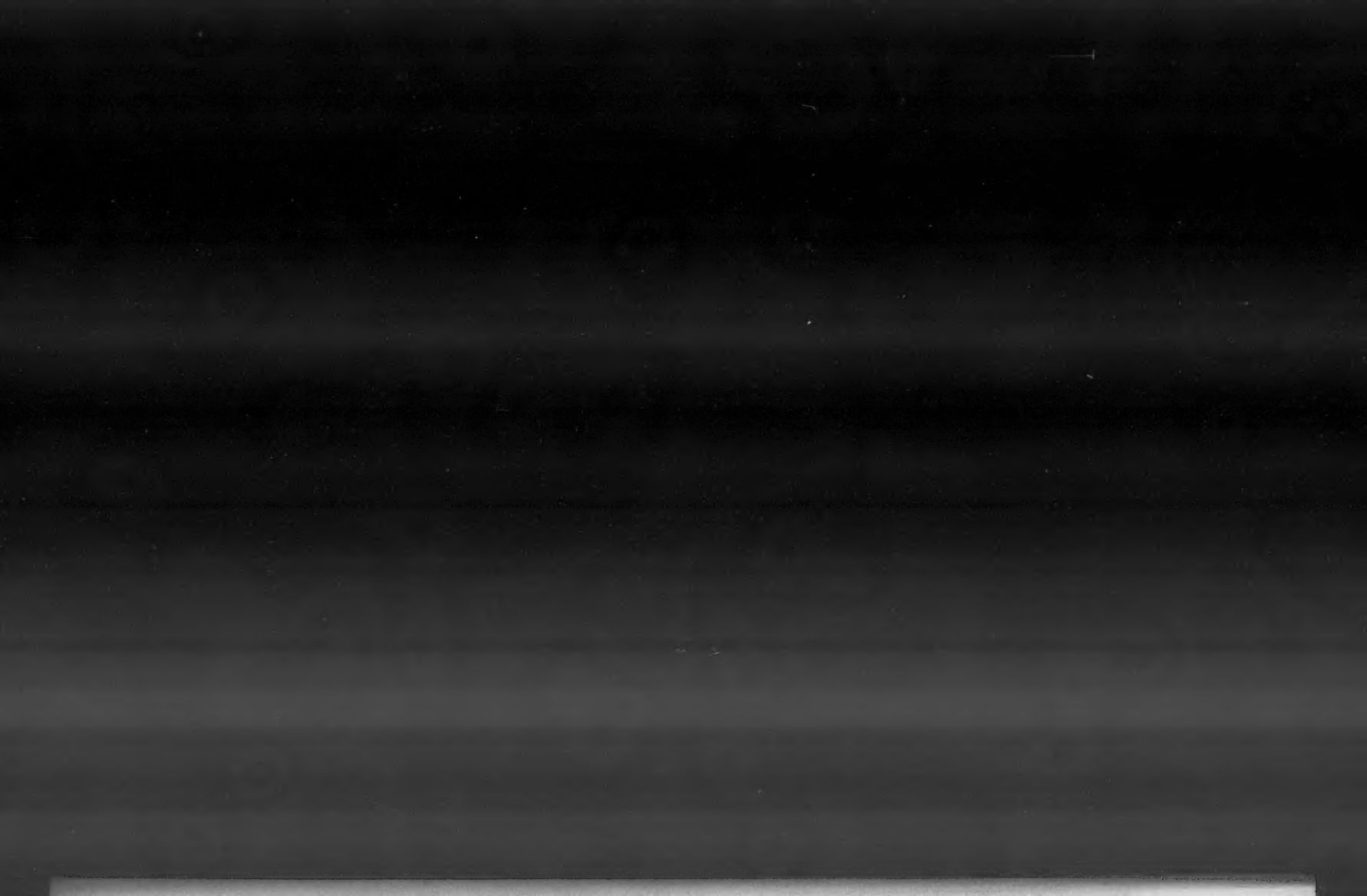
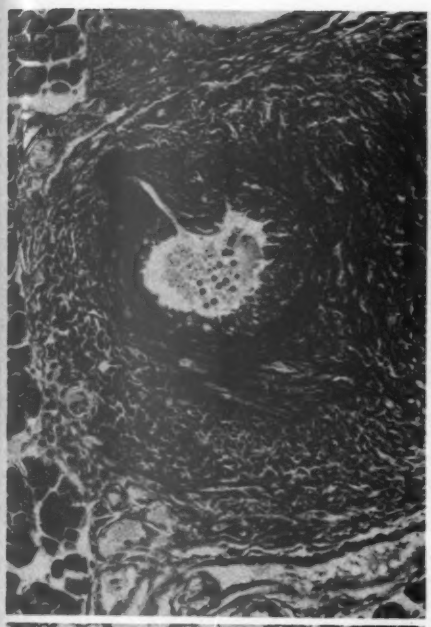
FIG. 7. Pancreatic artery showing slight thickening of walls and a slight inflammatory reaction. There is no apparent disruption of the architecture in the subendothelial region. Rat surviving renal infarction 1 month, treated with hydralazine. PAS stain. × 220.

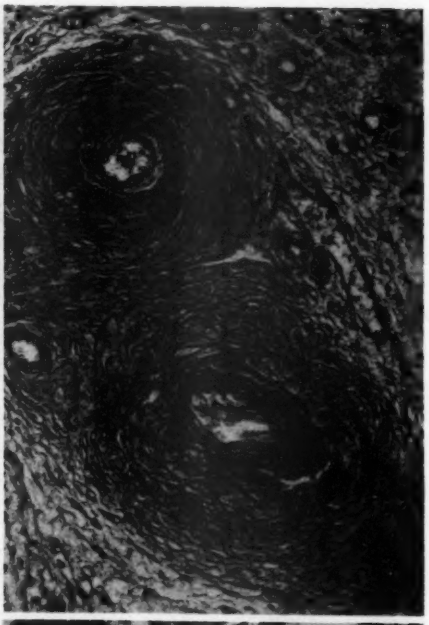
FIG. 8. Progressive lesions in two pancreatic arterioles characterized by continuous deposits of PAS-positive material which caused progressive narrowing of lumens. Rat treated with hydralazine for one month after renal infarction; no treatment for second month. PAS stain. × 220.

FIG. 9. Pancreatic artery, showing remission of vascular lesion. The markedly hypertrophied perivascular region is composed of sclerosed tissue in which dark spots representing blood pigment can be distinguished. The limit of the original vessel is presumably represented by the central core which stains darkly with PAS. It is relatively well repaired if one assumes that prior to treatment this artery was not unlike those in Figures 5 and 6. Rat with no treatment for one month after renal infarction; treated with hydralazine for second month before sacrifice. PAS stain. × 100.

FIG. 10. Small renal artery, showing progressive vascular alteration with deposition of PAS-positive material and thickening of the wall. Rat treated with hydralazine for two months after renal infarction. PAS stain. × 220.







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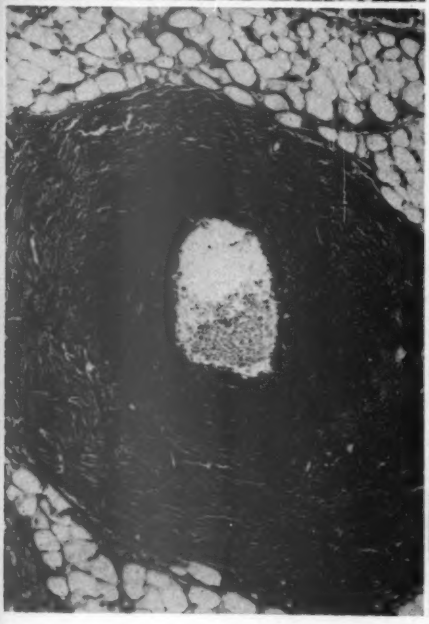

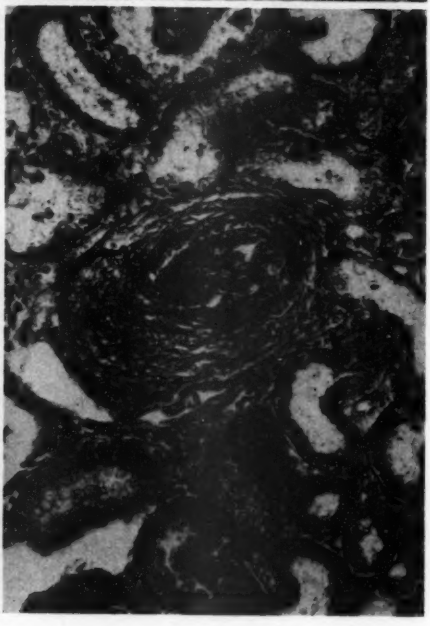

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HISTOLOGIC AND HISTOCHEMICAL STUDIES OF THE RABBIT GLOMERULONEPHRITIS PRODUCED BY SPECIFIC ANTI-KIDNEY DUCK SERUM*

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Glomerulonephritis can be induced in various animal species by specific anti-kidney serums. Its production by duck immune serum was first described in the rabbit by Masugi.[1] By using a potent standardized nephrotoxic duck serum, or its gamma globulin, nephritis can be reproduced in a regular manner. If sufficiently large amounts of serum are given, the course of the disease can be divided into 4 phases on histologic grounds: (1) There is an incubation period lasting for an average of 4 days following the initial dose of the nephrotoxic serum. (2) This is followed by the onset of an acute phase characterized by extensive glomerular alteration and the concomitant appearance of widespread hyaline droplet formation in proximal convoluted tubules and the appearance of tubular casts in distal portions of the nephrons. This stage lasts from the fifth to the seventh or eighth days following the injection. (3) The third phase, characterized by the progression of the glomerular lesion and marked tubular alteration, is accompanied by conspicuous reduction in the number of hyaline droplets. This phase lasts from the eighth to the 16th day. (4) A subacute and chronic stage reveals evidence of healing in many of the glomeruli.

In this easily reproducible renal disease, one can study the sequence of clinical and anatomic features from their onset to their termination within a few weeks. Thus the rabbit nephritis may serve as an experimental model for histologic and histochemical studies and as a basis for the evaluation of various therapeutic agents, such as cortisone[2] or heparin.[3]

Microscopic sections of kidney were studied with conventional and also with certain newer histochemical techniques. Special attention was paid to the tubular alterations which developed rapidly in this form of experimental glomerulonephritis.

Material and Methods

Ninety-two male albino rabbits of the New Zealand strain, weighing approximately 5 pounds, were used. All animals received Purina

* Supported by Research Grant A-688 (C) and A-322 (C) of the National Institutes of Health, United States Public Health Service, and by a grant from the Nephrosis Foundation of New York and New Jersey.

Received for publication, January 9, 1958.

rabbit pellets and water *ad libitum*. Large pools of immune duck serums, to insure uniformity of results, were produced as described elsewhere.[4] One cc. per pound of body weight was given intravenously on 2 successive days. Animals were sacrificed daily during the first days and at various time intervals up to 50 days following the initial sensitizing dose. Kidneys of animals that died spontaneously, usually during the second and third phases of the nephritis, were not used for microscopic study (Table I). Specific antibodies against the duck

TABLE I

Animals Examined in Various Periods

Days after injection of anti-rabbit-kidney duck serum	Number of animals		Renal specimens examined microscopically	
	Died spontaneously	Sacrificed	Conventional staining techniques	Enzymatic histochemistry
1 to 4	1	12	12	8
5 to 7	4	11	11	8
8 to 16	13	32	32	17
More than 16	0	19	19	6
Total	18	74	74	39

serum, complement, the total serum protein and albumin-globulin ratios, and cholesterol in the serum and urea nitrogen of whole blood were determined in experimental animals. The pertinent data, including observations in the urine, are reported in a separate communication.[4]

Thin slices of kidney were fixed in 10 per cent formalin and in Helly's and Rossman's[5] fluids. Paraffin sections were regularly stained with hematoxylin and eosin and the McManus periodic acid-Schiff (PAS) stain. Diastase and saliva controls were prepared on material fixed in Rossman's fluid when indicated. Sections were cut at 5 μ and at 1 to 2 μ. The following additional staining techniques were used: Sudan IV for lipid, Mallory's trichrome stain for connective tissue, Mallory's phosphotungstic acid-hematoxylin (PTAH) and iron hematoxylin stains on Helly-fixed material for the demonstration of mitochondria. For the evaluation of sulfhydryl groups, the technique of Barrnett and Seligman[6] was used on paraffin sections prepared from formalin-fixed tissue blocks. Amino groups were demonstrated with the techniques of Weiss, Tsou and Seligman,[7] using paraffin sections from Giemsa-fixed material. Tryptophan and related compounds were demonstrated with the method of Adams[8] on sections fixed in a mixture of 1 per cent trichloroacetic acid and 80 per cent ethyl alcohol. Sections from most kidneys were also stained with the Jones modification

of Gomori's silver-methenamine method.[9] In our hands, material fixed in Rossman's fluid gave the best results with the latter technique.

Enzymatic histochemistry was investigated in the kidneys of 39 animals (Table I), utilizing unfixed frozen sections and sections prepared from thin tissue blocks fixed in cold neutral formalin for 12 to 16 hours. A Sartorius freezing microtome with knife cooling was used for the preparation of these.[10] Sections were cut at 5 to 10 μ. The following enzymatic histochemical techniques were used: Nonspecific alkaline phosphatase, nonspecific phosphatase, 5-nucleotidase and adenosine triphosphatase at pH 7.2 were demonstrated in formalin-fixed[11,12] and glucose-6-phosphatase in fresh frozen sections.[13] For the demonstration of esterase, alpha naphthyl acetate served as substrate in both fresh and formalin-fixed tissues. For succinic dehydrogenase, the method of Seligman and Rutenburg was used with some modification.[14] Diphosphopyridine nucleotide (DPN) diaphorase was demonstrated by the technique of Farber, Sternberg and Dunlap.[15]

Results

Histologic Appearance of the Renal Lesions

First Phase. During the first phase, continuing to the fifth day after the initial injection of nephrotoxic serum, the rabbits appeared to be normal and the urine showed no albuminuria. In groups consisting of 3 animals each, sacrificed on the first, second, and third days, no significant microscopic lesions could be detected (Table II). On the fourth day, however, a distinct increase in the cellular content of glomerular tufts was noticed. This was confirmed when the cells within the glomeruli were counted. In normal controls and in the glomeruli of animals sacrificed during the first 3 days of the experiment, the average cell count was 30 per glomerulus; it rose to 45 on the fourth day. Most of the proliferating cells appeared to be endothelial. No changes were noted in the tubules stained with hematoxylin and eosin or the PAS technique. With silver stain,[9] occasional intracytoplasmic granules were seen in the epithelium of the proximal convoluted tubules. They were not digested by diastase. Similar granules were also noted in the normal controls.

Second Phase. During this phase, which continued from the fifth to the eighth days, clinical evidence of renal disease appeared. The animals became listless and often showed generalized edema. The earliest detectable manifestation was marked proteinuria. This was accompanied by an increase in blood urea nitrogen, a diminution of serum complement, and the appearance of specific anti-duck serum precipi-

TABLE II

Summary of Histologic and Histochemical Alterations

Days after injection of anti-rabbit-kidney duck serum	Glomeruli	Tubules	
		Histologic structure	Enzymatic histochemistry
1 to 4	Increase in endothelial cells (4th day only).	No lesions	No lesions
5 to 7	Proliferative, exudative and necrotizing lesions, "hyaline thrombi" in glomerular loops, and protein precipitates in Bowman's spaces. PAS positive; purple with PTAH stain; reacts with specific protein stains, but not with periodic acid, silver-methenamine. Basement membranes not thickened.	Extensive "hyaline droplet" formation in proximal convolutions. PAS and silver positive; react with stains for amino and sulphydryl groups and tryptophan. Diminution of mitochrondia in proximal convoluted tubules. Protein casts in distal tubules. Beginning degeneration and atrophy.	On 6th to 7th days, moderate diminution of alkaline phosphatase, 5-nucleotidase and glycerophosphatase at pH 7.2. Less depression of glucose-6-phosphatase and adenosine triphosphatase. No changes in esterase, succinic dehydrogenase and DPN diaphorase.
8 to 16	Proliferation of Bowman's capsules. Formation of PAS and silver positive fibrils. Thickening of basement membranes of glomerular capillaries and Bowman's capsules. Increased adenosine triphosphatase activity.	Marked atrophy accompanied by diminution in "hyaline droplets" and further reduction of mitochondria. Marked dilatation of distal tubules. Moderate fatty changes in proximal convoluted tubules. Focal thickening of tubular basement membranes.	Marked depression of all phosphatases, least noticeable with adenosine triphosphatase. Esterase and succinic dehydrogenase moderately depressed. DPN diaphorase least affected.
17 to 50	Concomitant repair and fibrous obliteration and hyalinization.	Mixture of normal, hypertrophic, and atrophic tubules.	Normal tubules show fairly normal enzymatic reactions. Almost complete disappearance in atrophic tubules, with the exception of DPN diaphorase which is only moderately inhibited.

tins. Four animals were sacrificed on the fifth day, 3 on the sixth, and 4 on the seventh day following the administration of nephrotoxic serum. All glomeruli were found to be affected (Table II). They were enlarged and showed both proliferative and exudative alterations. On the first day of proteinuria, proliferation prevailed (Fig. 1). Endothelial cells were increased markedly in number, and there was considerable obstruction of glomerular capillaries. Extracapillary cells had proliferated to a major degree within the stalk of the glomeruli. Glomerular epithelium was much less severely affected. The proliferating elements had comparatively large vesicular nuclei and a considerable amount of cytoplasm. Mitotic figures were rarely seen. Cells of Bowman's capsule were not altered during the initial two days of proteinuria. Occasionally, glomerular cells contained PAS-positive granules.

An exudative phenomenon consisted of the deposition of protein coagulum within the capillary lumens, forming so-called "hyaline thrombi." Protein material was also adherent to the capillary walls, frequently giving them a thickened, frayed and fluffy appearance. In sections cut at 1 to 2 μ and stained with either the PAS or the silver techniques, the basement membranes were not actually found to be thickened. This was best seen in sections in which no counterstain was used. The capillaries appeared enlarged, widened and sausage-shaped. As early as the second day of proteinuria, partial necrosis of capillary loops was noted. In such areas capillary membranes could not be demonstrated, and neutrophils were manifest. Frequently, there was extravasated blood from the necrotic loops free in Bowman's spaces. In affected glomeruli a diffuse proliferation of cells originating either from endothelial or epithelial components was often seen. Some of the cells contained bizarre nuclei. A few of the necrotic glomerular loops were replaced by irregular masses of protein material (Fig. 2). Protein coagulum was also present in Bowman's space in many instances in which the glomerular capillaries appeared intact. Abundant protein casts were present in the thin and thick limbs of Henle's loops and in collecting tubules. Only occasionally were the lumens dilated by these casts.

The epithelium of proximal convoluted tubules contained a striking number of "hyaline droplets." These appeared as granules, varying in size from 1 to 2 μ, which almost completely filled the cytoplasm of many cells (Fig. 3). The droplets were eosinophilic, stained red with the PAS and the Mallory stains, and had a brown to black hue with the silver method. They gave positive reactions for amino groups, sulfhydryl groups and tryptophan. In preparations stained with iron hema-

toxylin or PTAH, the droplets reacted positively while the rodlets of the mitochondria appeared in a state of dissolution (Fig. 4).

The intracapillary protein coagulum and that in both Bowman's capsule and the tubules reacted in similar fashion with the various protein stains, although the intensity was often less marked. This was particularly the case with the stain for sulfhydryl groups. With the PTAH stain, the protein material stained purple-blue and with the PAS stain, pink to red. With the silver stain, however, only a faint or negative reaction was obtained (Fig. 9). Jones has pointed out that in spite of the essential similarity in the principles of the staining techniques involved, certain structures reacted differently with the classical PAS and periodic acid-methenamine silver stains.[16] The PAS stain does not react with the collagen fibers, but does stain hyalin. In contrast, with the silver technique, collagen is stained but hyalin is not.

In the animals in which proteinuria had been present for more than two days, proliferation of the cells of Bowman's capsule was observed. In addition, the proximal convoluted tubules revealed changes not seen during the first two days. In some segments, epithelium filled with hyaline droplets became disintegrated. The droplets, which were often quite large, were discharged into the tubular lumens. Brush borders could not be demonstrated in such areas. In occasional instances there was a moderate amount of mononuclear cell infiltration, mainly in the vicinity of the glomeruli and larger cortical veins.

An additional observation was made in the kidneys of two animals. Within the epithelium of the excretory ducts near the papilla and in the pelvic epithelium covering the papilla, PAS and silver-positive granules were noted. These varied in size from dustlike particles to a diameter of one μ and usually appeared in the portion of the cytoplasm near the lumen. Variability in the location of the granules was attributed to artifacts of fixation. They did not react with any of the protein stains and could be digested by saliva or diastase. They were, therefore, composed of glycogen.

Third Phase. This phase, lasting from the eighth to the 16th days, was characterized by high levels of blood urea nitrogen and the severest degree of renal abnormality.

Five animals were sacrificed on the eighth day, 6 on the ninth day, and 6 on the tenth day. The glomerular alterations in all these animals were very prominent (Table II). There were necrotizing inflammation, glomerular hemorrhages, and extensive protein exudation into Bowman's spaces (Fig. 5). Cells of Bowman's capsule showed considerable focal proliferation. Protein coagulum lying within Bowman's space was invaded by proliferating cells which occasionally retained

connections with the capsule. The basement membranes of the glomeruli showed moderate focal thickening. The silver stain was particularly helpful in outlining this since adherent protein stained only very faintly. Distinct changes had occurred in the proximal convoluted tubules. Intracellular hyaline droplets were markedly reduced, but there was widespread extrusion of droplets into the lumens. Brush borders were often missing. The cytoplasm in some of the tubular epithelial elements appeared rarefied and empty. In others, there was a considerable decrease in the width of the cells themselves as well as of their brush borders. Thus, many tubular cells appeared atrophic (Fig. 5) and the lumens wider than normal. Stainable mitochondria were markedly decreased in number (Fig. 6). Occasionally, mitotic figures were seen in the epithelium of the proximal convoluted tubules. The extent of the atrophic change varied considerably in different animals. Glycogen deposition was observed in the excretory ducts of only two kidneys.

Fifteen animals were sacrificed from the eleventh to the 16th days. In these the proliferative and exudative changes in the glomeruli were diffuse and of severe degree. The deposition of protein in necrotic portions of glomeruli was very striking. In many instances marked proliferation of Bowman's capsule had occurred with formation of crescents and with partial obliteration of the periglomerular spaces. Between these proliferating cells, fibrils staining with the PAS and silver methods could be seen. Basement membranes of the glomeruli were often thickened (Figs. 9 and 10). The thickened capillary walls stained in a more homogeneous fashion with the PAS technique. With the silver technique, however, a double contour was often seen. In some instances, the intervening space was traversed by irregular, intertwined, silver-positive fibrils. The tubules in most kidneys showed marked atrophy, depletion of mitochondria and only occasional hyaline droplets. Occasionally there was considerable dilatation of distal tubules. Fat droplets were noted in a few proximal convoluted tubules in appropriately stained sections. Fatty alterations of this nature were not noted in earlier lesions. There was also discernible, by now, focal thickening of the capsular basement membranes about atrophic glomeruli.

Fourth Phase. This stage, which supervened after 16 days, was characterized by a tendency to partial spontaneous healing.

Seventeen animals were sacrificed between the 18th and 30th days following the administration of the nephrotoxic serum. In all kidneys, a varying number of glomeruli showed evidence of repair (Table II). Obstruction of capillary lumens by endothelial cells, evident in the

earlier stage, had now disappeared. The capillaries appeared dilated and congested. Protein precipitate adherent to the capillary wall was less conspicuous than in the earlier lesions.

Interspersed among glomeruli with more or less marked evidence of repair, were others which had lost function. In these the capsular basement membrane was thickened and the periglomerular space was filled with proliferating cells often concentrically arranged. At the periphery, these cells were not infrequently arranged in a pseudotubular fashion. PAS and silver-positive fibrils were seen between them. There was varying, but occasionally quite considerable, interstitial fibrosis, particularly in areas in which tubules with thickened basement membranes had become atrophic. Only occasional hyaline droplets and protein casts were seen.

Two animals were sacrificed after 50 days. The kidneys were of normal size, but one showed a slightly granular surface. Areas in which functioning glomeruli were clearly recognized alternated with others in which all glomeruli had become atrophic. In the vicinity of functioning glomeruli, hypertrophic tubules could be seen (Fig. 7). Thus the microscopic picture resembled that observed in subacute or chronic nephritis in man. The functioning glomeruli showed varying degrees of crescent formation with basement membrane thickening and focal fibrosis. Those which were atrophic often continued to exhibit proliferating cells, as in the earlier stages. Other glomeruli, however, were replaced completely by hyaline material. There was considerable fibrosis of parenchyma in the atrophic areas and marked interstitial infiltration by mononuclear cells. Basement membranes of atrophic tubules were greatly thickened.

Enzymatic Staining Reactions

The Normal Kidney. The distribution of succinic dehydrogenase in the rabbit is similar to that observed in the human kidney.[11] Strongest staining occurs in the ascending limbs of Henle's loops and within the cortex in segments which apparently correspond to the distal convoluted tubules (Fig. 8). Proximal convoluted tubules stain less strongly and the collecting ducts only irregularly and weakly. Diphosphopyridine nucleotide (DPN) diaphorase is active in all segments of the nephron. The terminal portions of the proximal convoluted tubules and collecting ducts give the strongest reaction. Staining is also observed in the thin limbs of Henle's loops and in the cells of Bowman's capsule and the capillary tufts. There is some staining in the walls of larger arteries. In frozen section preparations, esterase activity ap-

pears in all portions of the nephron. Activity is strongest in the proximal convoluted tubules and the thick limbs of Henle.

Alkaline phosphatase is demonstrable in the proximal convoluted tubules only; the staining reaction is of equal intensity in the proximal and distal segments (Fig. 11). Glycerophosphatase at pH 7.2 and 5-nucleotidase show a similar distribution pattern in fixed sections. Activity is strongest in the terminal portions of the convoluted tubules, and somewhat less so in their proximal portions. In unfixed sections, as pointed out previously,[11] a positive staining reaction is seen also in some tubules located in the medulla when the technique for glycerophosphatase is carried out at pH 7.2.

Glucose-6-phosphatase is visualized only in the proximal convoluted tubules (Fig. 12). Formalin fixation as used in the present study results in considerable inactivation of adenosine triphosphatase activity. In such sections this enzyme is manifest in the proximal convoluted tubules, localized mainly in the brush borders. There is some variation in the staining intensity in various segments of these tubules. The walls of all vessels located in the inner and outer medulla are regularly stained. The capillaries in the cortex as well as the glomeruli are nonreactive (Fig. 13). In unfixed sections, staining is also found in ascending limbs of Henle's loops, distal convoluted tubules, and some collecting tubules in the cortex. The cells of the ascending limbs of Henle's loops and the distal convoluted tubules show perpendicular striations in their cytoplasm, simulating the arrangement of mitochondrial rodlets. Dr. A. B. Novikoff has drawn our attention to the fact that these striations apparently correspond to infoldings of the cellular membranes. The mitochondria have no demonstrable adenosine triphosphatase activity.

The Nephritic Kidney (Table II). No alterations in any of the staining reactions occurred during the incubation period.

On the first day of marked proteinuria, in the second phase, no alterations were noted. Of 2 animals sacrificed on the second day, one showed mild changes. In 4 animals sacrificed on the third and fourth days of proteinuria, similar mild lesions were seen. They were noted most clearly in sections stained for alkaline phosphatase. There was distinct focal diminution of the staining in some of the proximal convoluted tubules best seen in sections that had been incubated for 10 to 15 minutes. Occasional tubules showed diffuse staining for the most part in the distal portions. This was quite different from the brush border staining usually seen in these preparations. Similar changes were noted in sections stained for 5-nucleotidase and glycerophospha-

tase at pH 7.2. A slight, but less consistent diminution of activity was observed in sections prepared for the demonstration of glucose-6-phosphatase and even less consistent reduction occurred in those stained with the adenosine triphosphatase technique. Esterase, DPN diaphorase, and succinic dehydrogenase activities were essentially unchanged except in one animal in which mild reduction of succinic dehydrogenase was found.

During the third phase, in 17 animals sacrificed between the ninth and 16th days, there was marked and generalized depression of alkaline phosphatase activity. This was more noticeable proximally in the proximal convolutions than distally (Fig. 14). Marked diminution of activity was also seen in sections stained for glycerophosphatase at pH 7.2 and in those demonstrating 5-nucleotidase. The reduction was less manifest when the adenosine triphosphatase technique was used. While in normal animals, glomeruli were nonreactive when stained for adenosine triphosphatase, there was distinct activity in the capillary walls of the damaged glomeruli (Fig. 15). Esterase was less influenced in general although its activity was depressed in some of the kidneys. Succinic dehydrogenase was reduced to a lesser degree and only in the proximal convoluted tubules while the intense staining reaction of ascending limbs of Henle's loops was not diminished (Fig. 16). At this stage DPN diaphorase was the least affected of the various enzymatic staining reactions tested (Fig. 17). There was only mild reduction of staining in the proximal convoluted tubles. It was noteworthy that proliferating elements within both the tufts and Bowman's capsules showed a distinct cytoplasmic staining reaction. The staining reaction for esterase and DPN diaphorase in the collecting ducts and thin limbs of Henle's loops showed no significant alterations.

Six animals were examined during the 20 to 50 day period of the disease (the fourth phase). Corresponding to the lesions in routine sections, reduction of enzymatic activity was essentially focal in nature. Wherever functioning glomeruli were surrounded by functioning tubules, the tubules showed normal histochemical staining reactions. Atrophic tubules exhibited no activity (Fig. 18) except that indicative of DPN diaphorase.

Discussion

Comparatively few reports dealing with rabbit nephritis induced by avian immune serums have appeared since Masugi[17-20] described the production of nephrotoxic nephritis in the rabbit almost 25 years ago. With the potent serums used in this investigation, it has been possible to produce a severe glomerulonephritis consistently. This experimental disease lends itself particularly well to study of its acute phase. Ani-

mals which survived the acute stage developed a subacute and chronic form of renal disorder. Thus the experimental disease can serve as an ideal model for the investigation of immunologically induced renal lesions and the influence of therapeutic measures upon them. The use of the rabbit also permits the procurement of larger blood samples for the study of biochemical changes. It should be stressed, however, that a disease as severe as that described in the present communication will only result when relatively large doses of potent nephrotoxic serum are introduced. With smaller amounts, much milder lesions are induced with only transient elevations of blood urea nitrogen and with marked tendency to spontaneous healing.

It was pointed out by Masugi that rabbit nephritis, in contrast to that in the rat, only occurred after an incubation period of several days. With the use of our serums, this latent period was almost regularly of 5 days' duration. On the fourth day, about 24 hours preceding the onset of albuminuria, an increase in the number of glomerular elements was found in the sacrificed animals. The morphologic manifestation of glomerular involvement in the initial stages was of both a proliferative and exudative character. Endothelial cells were greatly increased in number and contributed to the obstruction and ischemia of the glomerular loops. Epithelial elements as well as cells located in the glomerular stalk participated in the tissue reaction. It is not intended to discuss here the hotly debated question of the existence of mesangial cells in the normal and abnormal glomeruli. Reference to this matter may be found in several recent papers.[21-24] Suffice it to say that, in general, workers using the conventional microscope favor the existence of the mesangium while most electron microscopists are not able to identify it with certainty.

Deposition of so-called hyaline thrombi within glomerular capillaries was observed on the first day of proteinuria. The thrombi contributed to the obstruction of the capillary lumens. Occasionally, the deposition resembled the renal lesion in the generalized Shwartzman reaction in the rabbit.[25] While the administration of nephrotoxic serums does not produce such changes in the rat, the additional administration of endotoxins derived from gram-negative bacilli can elicit a similar tissue response.[26]

Exudation of protein also appeared in Bowman's capsular spaces and in the renal tubules. The severest form of tissue insult was characterized by the widespread necrosis of capillary loops. These necrotic lesions often contained PAS-positive material with a positive reaction to various specific protein stains. The substance also resembled fibrin in staining character, with the PTAH stain. Although capillary walls

appeared thickened and fluffy because of adherent protein precipitate, specific stains showed no thickening of the basement membranes at the onset of the disease.

During succeeding days, the accumulation of protein in glomerular capillaries, basement membranes and Bowman's spaces became very prominent. The appearance of similar material in the glomeruli has also been described in the nephritis induced in rabbits by the administration of foreign protein.[27] Teilum, Engbaek, Harboe and Simonsen[28] have produced subacute and chronic nephritis in rabbits by repeated injections of Pfeiffer bacillus culture and have likewise observed similar protein deposits in glomeruli. They considered the substance to be of the nature of hyalin. The deposits were interpreted by them to be products of secretion by endothelial cells. The obvious origin of similar material from exuded plasma protein in the rapidly developing nephritis we have studied does not support their contention.

Thickening of the basement membranes in damaged glomeruli developed after a few days of the violent clinical disease. In thin sections stained with periodic silver-methenamine, double contours with fine intervening fibrils could be seen. In more chronic cases, diffuse thickening of basement membranes occurred. There was also an increased density in the glomerular stalk reminiscent of the scarring observed in advanced human nephrosis by Jones.[9] Recent studies with the electron microscope in biopsy material from patients with nephrosis emphasize the changes in epithelial cells; alterations in basement membranes are only slight. In cases of acute nephritis, proliferation and swelling of endothelial cells occurs, and there is an accumulation of material resembling basement membrane in density and general appearance. This material appears as bars and irregularly shaped strands interlaced with endothelial cytoplasm. The electron microscopic appearance suggests that it is synthesized as a result of endothelial activity.[29-31]

Animals which survived 16 days following the injection of nephrotoxic serum showed signs of repair of glomeruli. This was evidenced by the disappearance of glomerular obstruction and obvious re-establishment of glomerular blood flow. At a later date, some of the kidneys showed microscopic lesions reminiscent of subacute and chronic glomerulonephritis in man. Groups of more or less functional glomeruli associated with functioning tubules alternated with foci of interstitial fibrosis. In the fibrotic areas, glomeruli were either atrophic or completely replaced by proliferating endothelial cells. Associated tubules were completely atrophic.

The initial tubular changes occurred when proteinuria became mani-

fest. Many cells of the proximal convoluted tubules contained "hyaline droplets." These, on the basis of all staining reactions, represented protein absorption droplets. Their production experimentally, following the administration of various types of proteins, and their histologic and histochemical characteristics have been extensively studied in the rat by Oliver, Moses, MacDowell and Lee.[32,33] In appropriately stained sections of rabbit kidneys in the early phases of nephritis, the disappearance of the normal mitochondrial rodlets and the appearance of positively staining absorption droplets could be observed in a manner similar to that described by Oliver and co-workers. Simultaneously with their appearance, protein casts were deposited in many of the distal tubules. Within a few days, the protein droplets were decreased in number considerably and were seen to be extruded into the tubular lumens. The extrusion was associated with considerable cellular damage and distortion of the brush borders. Soon thereafter, many proximal convoluted tubules appeared atrophic, showing loss of stainable mitochondria, narrowed cytoplasmic width, and narrowed brush borders.

An interesting phenomenon observed in several of the kidneys in the earlier stages of the disease was the accumulation of glycogen in the most centrally located portions of the excretory ducts, and the covering epithelium of the renal papilla. The occurrence of glycogen in this location has been described previously by Hinde[34] in newborn animals of various species, but most consistently in the rabbit. We are not aware of selective glycogen accumulation in this particular area of the kidney in any other abnormal condition in adult animals.*

The striking changes in the appearance of the proximal convoluted tubules suggested that a study of various enzymatic histochemical staining reactions be made. As has been indicated previously,[11] these staining reactions occur in the normal mammalian kidney in a regular and reproducible way. No alterations in the staining reactions were noted during the incubation period preceding the nephritis. Changes became noticeable on the second and third days of marked proteinuria (at the end of the second phase), and became very pronounced during the third phase. The most sensitive staining reaction was that for alkaline phosphatase, and almost as sensitive in indicating early lesions were the techniques for 5-nucleotidase and glycerophosphatase at pH

* Since this paper has been submitted for publication, glycogen has been described as a regular occurrence in the collecting renal tubular epithelium of two-month-old rabbits. (Spicer, S. S. Histologic localization of glycogen in the urinary tract and lung. *J. Histochem.*, 1958, 6, 52–60.) Thus it would appear that this constitutes a physiologic event in the rabbit.

7.2. Reduction of these enzymatic staining reactions was only slight and focal in the beginning. With the progression of atrophic changes, inactivation of stainable phosphatases became very striking. Only slightly less sensitive was the procedure for glucose-6-phosphatase; the least sensitive of the specific phosphatases was adenosine triphosphatase. Esterase activity was diminished only when atrophic changes in the tubules were quite noticeable. Similarly, succinic dehydrogenase activity was not appreciably changed in the earlier phases of nephritis. Of all enzymes tested, DPN diaphorase was the least affected. Partial reduction of its activity was seen only in very atrophic tubules. It is noteworthy that all of the changes observed were limited to the proximal convoluted tubules. However, an increase in activity was noted in the glomeruli with the DPN diaphorase method and to a more marked degree with the adenosine triphosphatase technique.

Recently an apparent shift of alkaline phosphatase activity from the positive-reacting proximal convoluted tubules to the diseased glomeruli has been described in nephrotoxic nephritis in rats.[35] Such a phenomenon was not observed in our animals. However, we did note a somewhat similar feature, manifested by a marked increase in glomerular triphosphatase activity accompanied by an obviously independent decrease in tubular activity. Fisher and Gruhn[36] studied a number of histochemical staining reactions in experimentally induced renal disease in the rat. They found no changes in lipase, nonspecific esterase, alkaline and acid phosphatase, glucose-6-phosphatase or 5-nucleotidase. Only succinic dehydrogenase and cytochrome oxidase activities were diminished. These observations, which differ considerably from those we have encountered in the rabbit, require further clarification.

A comparison of sections prepared with conventional stains with those stained by histochemical enzyme techniques showed no consistent evidence that diminution of enzymatic activity preceded the appearance of detectable lesions in routine sections. In the kidneys of only one animal, sacrificed on the second day of the nephritis, sections stained by various conventional methods revealed essentially normal structure; in appropriately stained sections, some of the enzymes were found to be reduced in activity.

One of the interesting observations related to the application of histochemical staining reactions in various forms of tubular damage is the recognition of a difference in the sensitivity of various enzymes, dependent upon the nature of the injury. Succinic dehydrogenase disappears very early in the toxic damage following the administration of Mercuhydrin, while even completely necrotic cells retain considerable phosphatase activity. In ischemic necrosis following arterial liga-

tion, succinic dehydrogenase is quite stable while adenosine triphosphatase and glucose-6-phosphatase are affected at an early date.[37]

The first experimental procedure in which the alkaline phosphatase reaction was studied was ligation of the ureter. Wilmer[38] found noticeable reduction of the enzyme in rabbits and rats within a few days following this procedure, and believed that disappearance of phosphatase preceded any other demonstrable alteration. We were able to confirm Wilmer's results, but could not detect any diminution of stainable alkaline phosphatase in advance of manifest alterations in conventionally stained sections.[39] Later, succinic dehydrogenase was found to be less affected by experimental hydronephrosis than alkaline phosphatase.[11,14] It would thus appear that the alterations in rabbit nephritis resemble those in experimental hydronephrosis, but are distinctly different from those accompanying toxic or anoxic damage.

One of the most intriguing aspects of experimental rabbit nephritis concerns the mechanism of the tubular damage. Three possibilities might be considered: (1) The tubular damage is attributable to an antibody reaction occurring in both the glomeruli and the tubules. However, fluorescent antibodies are seen only in the glomeruli of rabbits with glomerulonephritis induced by injections of purified foreign proteins.[40] One must also take into account the fact that the tissue response is both proliferative and exudative in the glomeruli while the reaction in the tubules is purely degenerative. (2) The overloading of the proximal convoluted tubules with reabsorbed protein may damage tubular epithelium to such a degree that tubular atrophy ensues. (3) Mechanical blockage of the distal tubules by the rather massive and widespread protein casts may result in the creation of an internal hydronephrosis.

The microscopic observation of destruction of brush borders and the disintegration of epithelium with disgorgement of protein granules into the tubular lumens seems to support the second possibility. Both factors, tubular blockage by protein casts as well as tubular damage characterized by protein granule formation, could explain the tubular atrophy and both the concomitant and subsequent alteration in histochemical staining reactions. Attempts are under way to further evaluate the factor of protein leakage and its significance in the genesis of tubular damage in experimental nephritis.

Summary

1. A reproducible glomerulonephritis was induced in rabbits by the administration of potent anti-rabbit duck serum. The disease was accompanied by typical biochemical features: proteinuria, increase in

blood urea nitrogen and blood cholesterol, and decrease in serum albumin. There was also decrease in complement and increase in specific anti-duck serum precipitins.

2. On morphologic grounds, this disorder was divisible into 4 stages: an incubation period continuing for approximately 5 days; a proliferative and exudative phase, lasting from the fifth to the eighth days; a third phase from the eighth to the 16th days with progression of glomerular lesions and tubular atrophy, and a fourth stage observed from the 16th day to 50th day in which evidence of repair was apparent.

3. Histochemical enzymatic staining reactions revealed a regular staining pattern in normal controls. In the kidneys of experimental animals, the earlest reduction of activity was noted in sections stained for alkaline phosphatase, glycerophosphatase at pH 7.2 and 5-nucleotidase. The technique for glucose-6-phosphatase was slightly less sensitive. Adenosine triphosphatase and esterase, as well as succinic dehydrogenase, revealed diminished activity only in later phases. DPN diaphorase appeared to be the least sensitive enzyme. In general, depression of histochemical enzymatic activity paralleled the severity of tubular atrophy.

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LEGENDS FOR FIGURES

FIG. 1. Rabbit sacrificed on the first day of proteinuria. Marked increase in glomerular cellularity with blockage of capillary lumens and several "hyaline thrombi" in upper glomerulus. Periodic acid-Schiff (PAS) and hematoxylin stain. × 200.

FIG. 2. Rabbit sacrificed on the second day of proteinuria. Note protein deposition in necrotic glomerular loop. A multinucleated irregular cell mass is seen in the vicinity. PAS and hematoxylin stain. × 250.

FIG. 3. Proximal convoluted tubules show extensive deposition of "hyaline droplets" in the kidney of a rabbit sacrificed on the second day of proteinuria. PAS and hematoxylin stain. × 400.

FIG. 4. The cells of the centrally located convoluted tubule contain protein absorption droplets and mitochondria in various stages of dissolution (second day of proteinuria). Phosphotungstic acid hematoxylin (PTAH) stain. × 500.

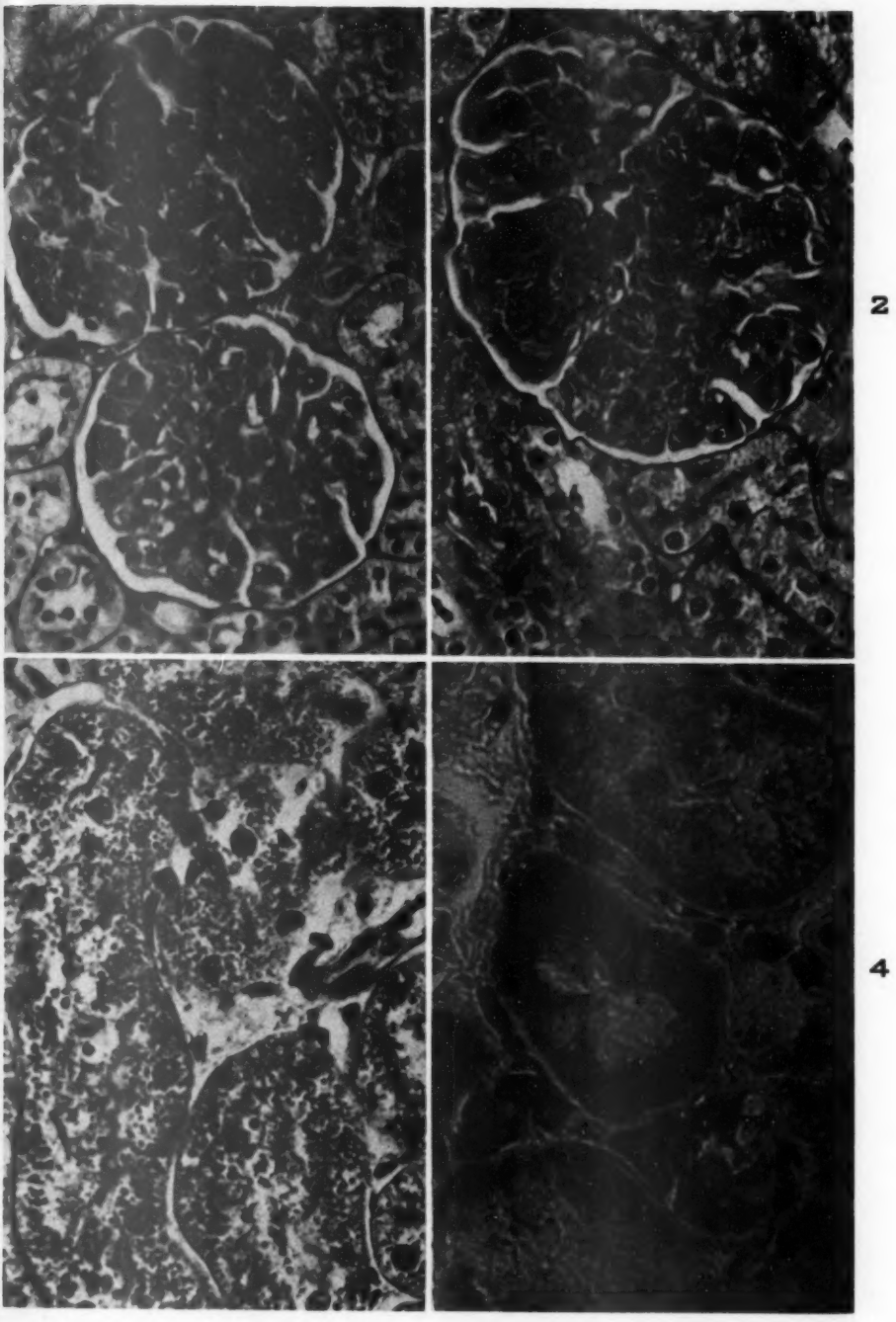
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FIG. 5. Rabbit sacrificed after 5 days of proteinuria. Glomeruli show extensive protein deposits in capillaries and Bowman's spaces. Tubules exhibit atrophic changes. PAS and hematoxylin stain. × 200.

FIG. 6. Rabbit sacrificed after 4 days of proteinuria. The number of stainable mitochondria is markedly reduced in most of the proximal convoluted tubules. PTAH stain. × 200.

FIG. 7. Rabbit sacrificed after 45 days of experimental glomerular nephritis. The microscopic picture resembles that of "chronic" glomerulonephritis in man. PAS and hematoxylin stain. × 75.

FIG. 8. Fresh frozen section of normal rabbit kidney, stained for succinic dehydrogenase activity. The most intense staining is seen in distal convoluted tubules. Glomeruli do not react. Incubation time, 120 minutes. In this and all following illustrations no counterstain was used. × 150.

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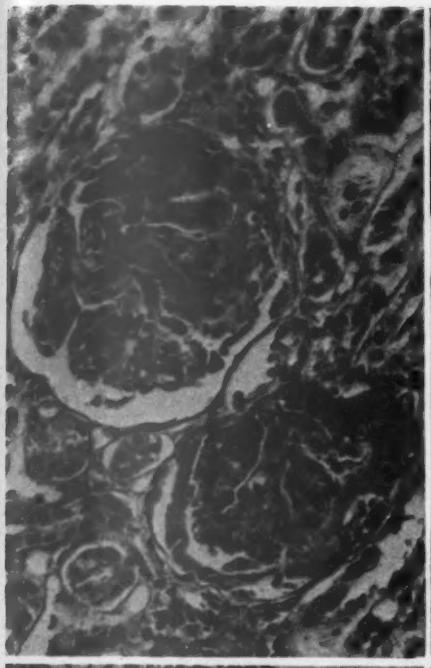

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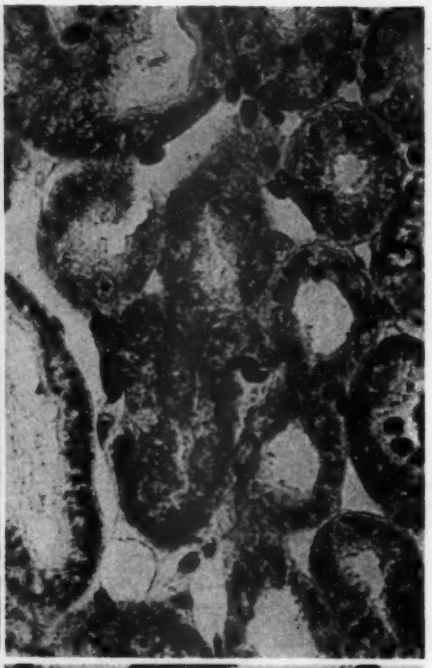

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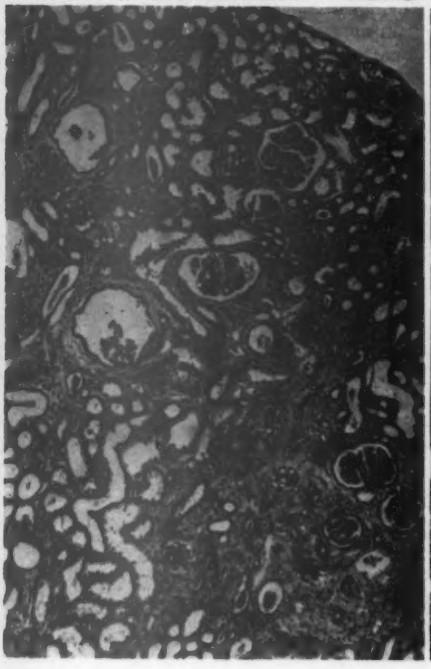

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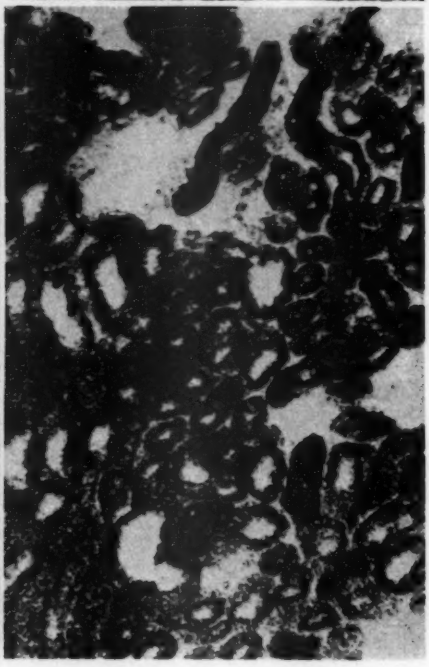

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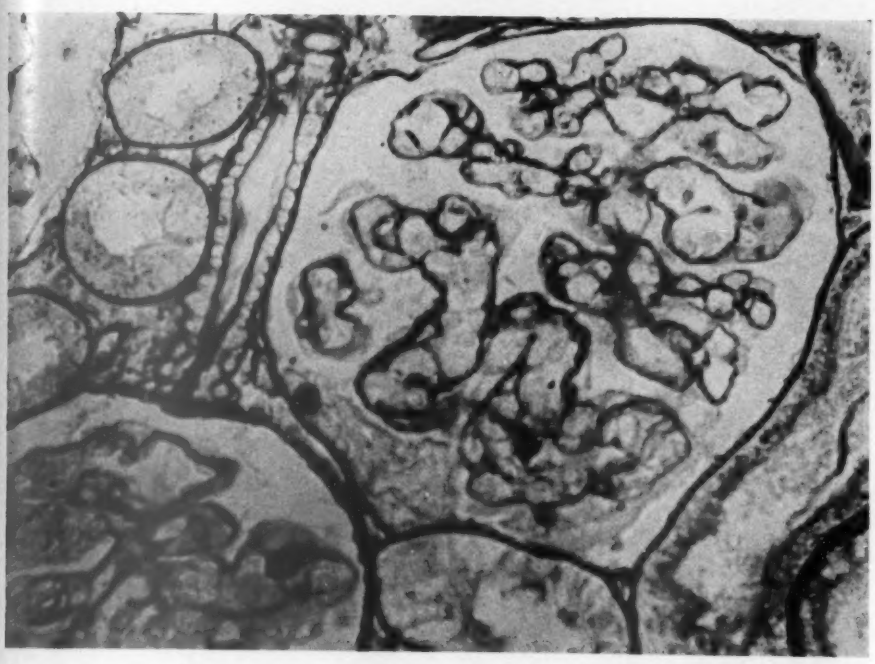
FIG. 9. Rabbit sacrificed on the fourth day of proteinuria. Capillary loops appear widened and show focal thickening of their basement membranes. The outlines of an unstained protein coagulum are seen in the lower portion of Bowman's space in the upper glomerulus. Jones periodic acid silver-methenamine technique. × 700.

FIG. 10. A higher magnification of part of the upper glomerulus shown in Figure 9. Note the focal thickening of basement membranes with double contours and formation of silver-positive fibrils. Jones periodic acid silver-methenamine technique. × 1,400.



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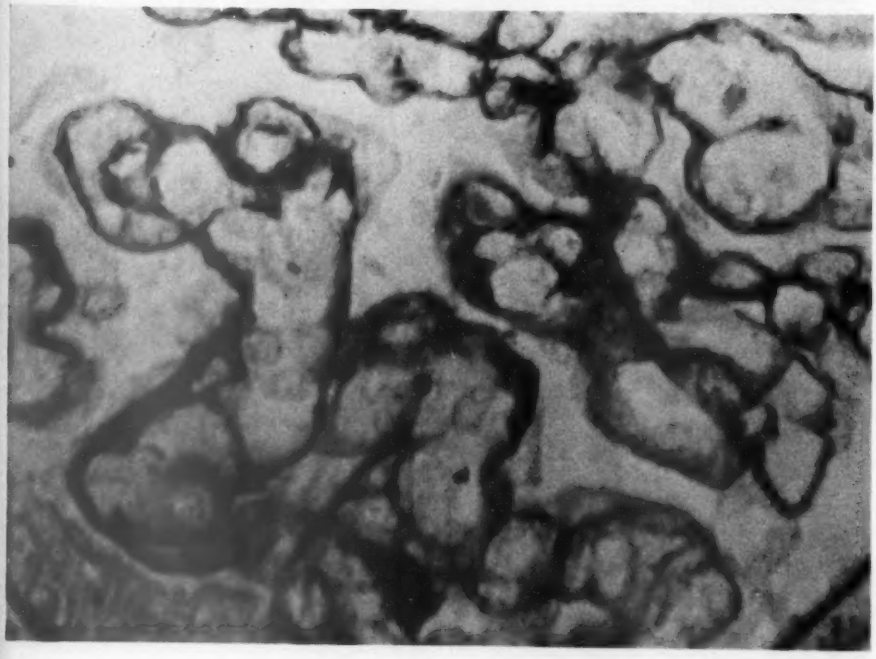

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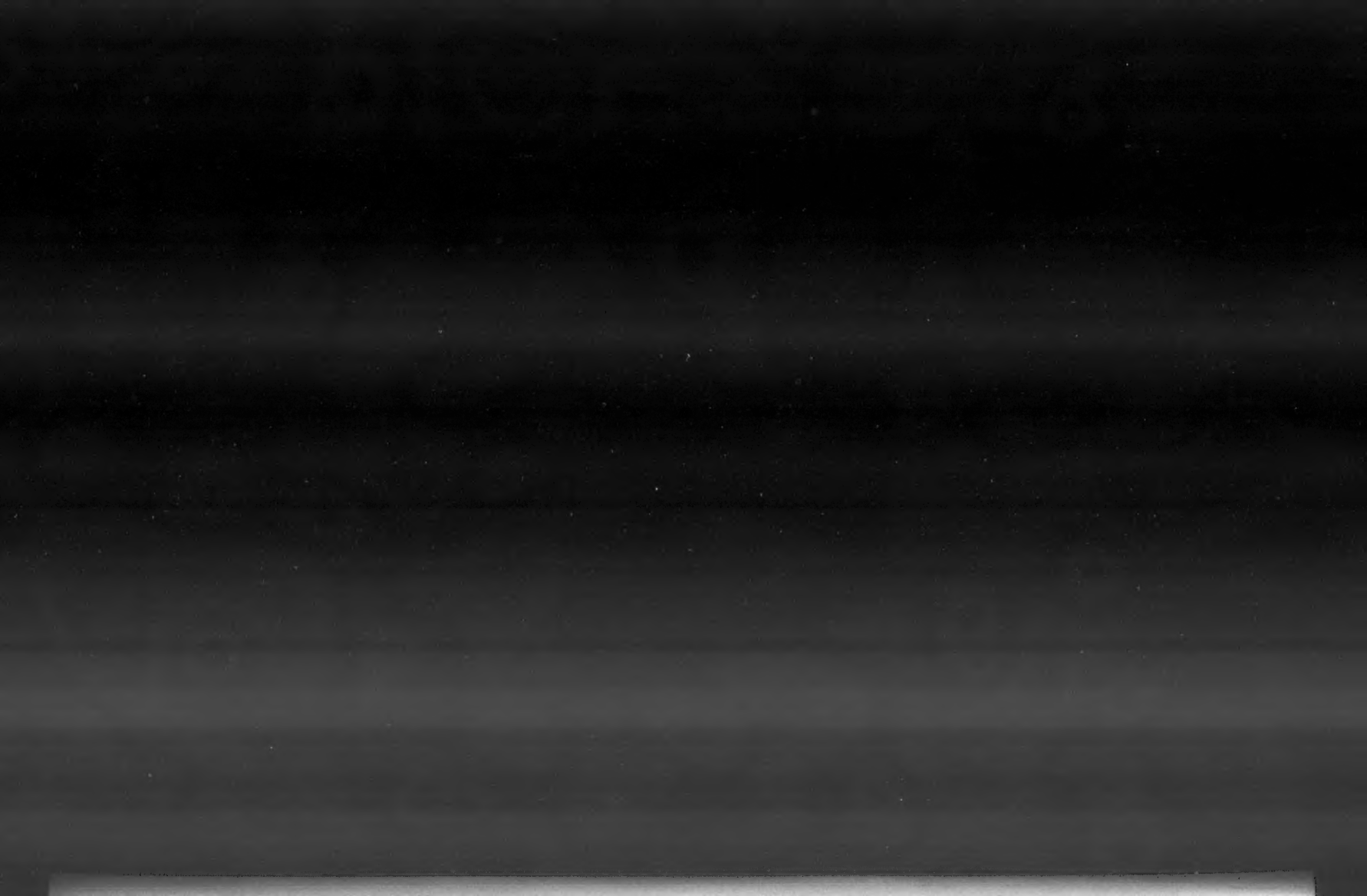
FIG. 11. Formalin-fixed frozen section from the kidney of a normal control rabbit, stained for nonspecific alkaline phosphatase. Proximal convoluted tubules react intensely. Incubation time, 15 minutes. × 150.

FIG. 12. Fresh frozen section from the kidney of a normal control rabbit, stained for glucose-6-phosphatase. The proximal convoluted tubules show intense staining. Incubation time, 30 minutes. × 100.

FIG. 13. Formalin-fixed frozen section from the kidney of a normal control rabbit, stained for adenosine triphosphatase activity. Proximal convoluted tubules show marked staining of brush borders, but glomeruli are not reactive. Incubation time, 30 minutes. × 250.

FIG. 14. Formalin-fixed frozen section from the kidney of a rabbit after 8 days of proteinuria, stained for nonspecific alkaline phosphatase. There is very extensive inactivation of enzymatic activity. Incubation time, 30 minutes. × 150.



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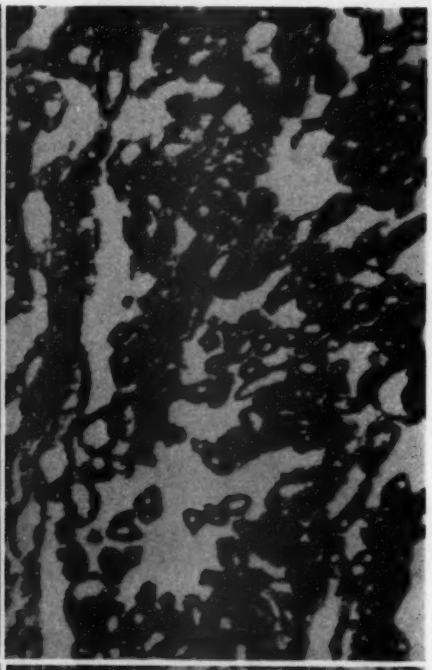

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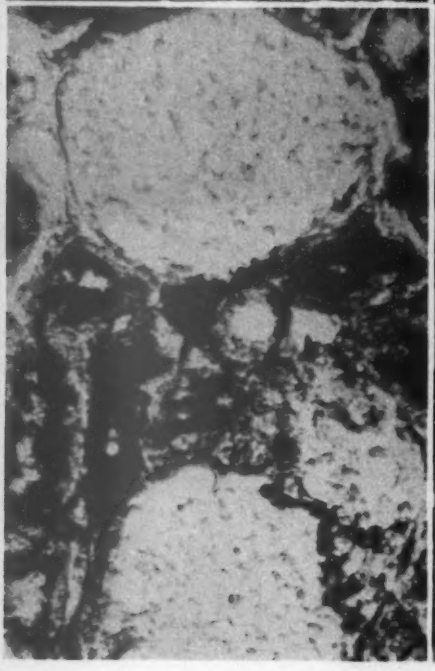

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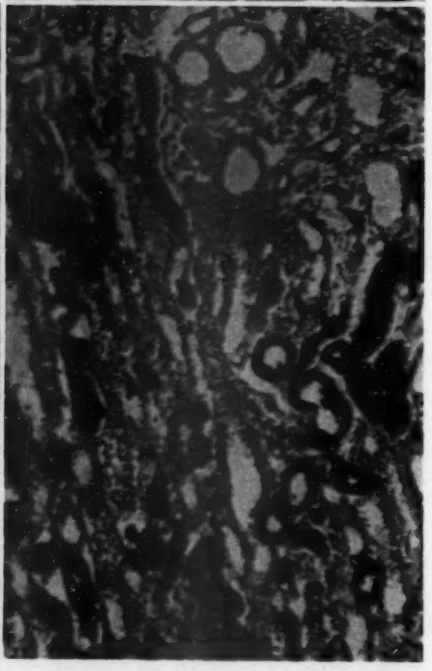

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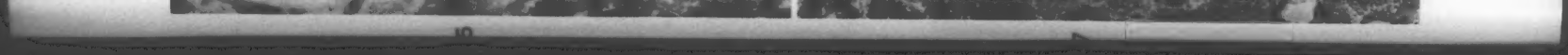
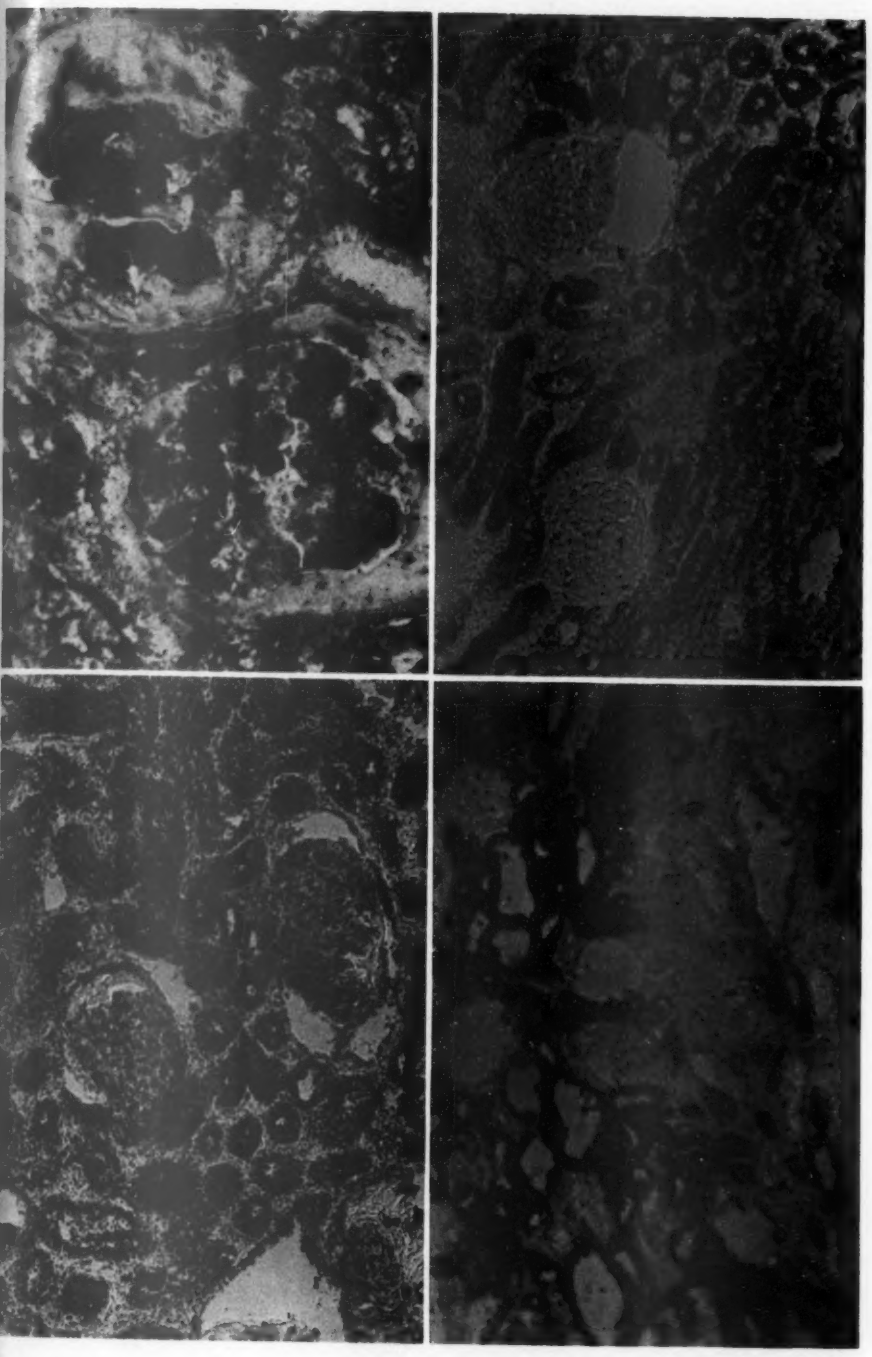
FIG. 15. Formalin-fixed frozen section from the kidney of a rabbit after 6 days of proteinuria, stained for adenosine triphosphatase activity. Compare with Figure 13. There is striking activity in the glomerular capillaries which are surrounded by nonreactive protein coagulum. Activity in proximal convoluted tubules is diminished. Incubation time, 30 minutes. × 250.

FIG. 16. Fresh frozen section from the kidney shown in Figure 14, stained for succinic dehydrogenase. Compare with Figure 8. There is moderate diminution of enzymatic staining in proximal convoluted tubules. Incubation time, 120 minutes. × 150.

FIG. 17. Fresh frozen section from kidney shown in Figures 14 and 16, stained for DPN diaphorase activity. There is very little diminution in the intensity of the staining reaction in cortical tubules as compared to normal controls. Note considerable activity in glomerular cells. Incubation time, 120 minutes. × 150.

FIG. 18. Fresh frozen section from the kidney of a rabbit after 44 days of glomerulonephritis, stained for glucose-6-phosphatase activity. Compare with Figure 12. Functioning, enzymatically active tubules are interspersed among atrophic nonreacting nephrons. Incubation time, 30 minutes. × 100.





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THE JUXTAGLOMERULAR CELLS IN MAN AND THEIR RELATIONSHIP TO THE LEVEL OF PLASMA SODIUM AND TO THE ZONA GLOMERULOSA OF THE ADRENAL CORTEX *

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A relation between levels of plasma sodium and potassium and the adrenal cortex has been demonstrated in both animals and man. Evidence has indicated that the zona glomerulosa of the adrenal cortex is concerned specifically with the production of mineralo-corticoids. Adrenocorticotrophic hormone (ACTH) primarily affects the zona fasciculata but an analagous trophic hormone has not been found for the zona glomerulosa.[1-3]

Dunihue has demonstrated hypergranulation of the juxtaglomerular cells of the kidney in adrenalectomized animals.[4,5] The Hartrofts have shown a correlation in rats and dogs between levels of dietary sodium, the degree of granulation of the juxtaglomerular cells, and the width of zona glomerulosa of the adrenal cortex.[6,7] Therefore, adrenals and kidneys obtained at necropsy were examined to see if any of the above relationships might be demonstrated in man. Previous work on the juxtaglomerular apparatus in man has emphasized changes associated with various disease entities.[8-10]

MATERIALS AND METHODS

Kidneys from an unselected series of 200 necropsied patients were sectioned and stained by a modification of the Bowie technique, as described by Cowdry,[11] in order to demonstrate granules in the juxtaglomerular cells. The degree of granulation of the juxtaglomerular cells was determined by a semiquantitative method previously described.[9] This procedure yields a juxtaglomerular granulation index (JGI) which can be correlated with clinical and other pathologic findings.

Staining Technique

Thin slices of tissue are fixed in Helly's fluid (Zenker's solution without acetic acid but with addition of 5 cc. of formalin per 100 cc. immediately before use) for 48 hours and washed in running tap water

* Supported by a grant from the Nutrition Foundation, Inc., New York, N.Y., and Grant A-1114 from the U.S. Public Health Service, Bethesda, Md.

Received for publication, January 17, 1958.

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for 24 hours. Specimens are mounted in paraffin blocks which may be trimmed and treated several hours with Mollifex (The British Drug Houses, Ltd.) in order to soften the tissue. Sections should not be more than 4 μ in thickness. If albumen is used as an adhesive, care should be taken to avoid excessive amounts, as it may interfere with the staining.

Preparation of Bowie's Stock Solution. Dissolve one gram of Biebrich scarlet in 250 cc. of distilled water and filter through a rapid filter paper into a beaker. Dissolve 2 gm. of ethyl violet in 500 cc. of distilled water and filter a small amount at a time into the same beaker with frequent stirring. The end point of neutralization is indicated when a small amount of the mixture placed on a filter paper does not show any color (other than the precipitate itself). The mixture should then be filtered and the precipitate dried. The stock solution is made by dissolving 0.2 gm. of the dried precipitate in 20 cc. of 95 per cent alcohol.[11] Up to 100 cc. of stock solution can be obtained from one batch if the end point is carefully determined.

Staining Procedure. (1) Take paraffin sections rapidly through xylols and alcohols to alcoholic iodine. Immerse for not more than 3 minutes in iodine and 3 minutes in sodium thiosulfate. Wash in running tap water for 5 minutes. (2) Mordant in 2.5 per cent potassium bichromate at approximately 40° C. over night. (3) Rinse with distilled water and immerse sections over night in 20 per cent ethyl alcohol to which has been added 10 to 15 drops of Bowie's stock solution per 100 cc. (4) Blot sections with bibulous paper. (5) Dip quickly 2 to 3 times in 2 changes of acetone to remove excess stain. (6) Differentiate in a 1:1 mixture of xylol and clove oil until section appears red or reddish purple. Microscopically, renal parenchyma should be red (or magenta) in contrast to elastic tissue of vessels which should be blue-purple. Juxtaglomerular cell granules, where present, will be the same color as elastic tissue. The latter provides a convenient criterion for determining completion of differentiation. Red blood cells are usually amber as a result of previous bichromate mordanting. (7) Rinse in 2 changes of xylol followed by 2 changes of benzene. Mount with benzene balsam or Permount.

Selection of Cases

The clinical charts of the 200 patients were examined, and data concerning levels of blood pressure, electrolyte determinations, age, race, and sex were extracted. Of these 200 cases, 24 had had at least 3 determinations of the level of plasma sodium within the last week of life. These 24 cases form the chief basis of this report, as fewer than 3

TABLE I

Summary of Data from 24 Patients

Case number	JGI*	Width(μ) zona glomerulosa	Serum sodium	Serum potassium	NPN (BUN)†	Age	Race, Sex	Heart wt. (gm.)	Kidney wt. (gm.)	Adrenal wt. (gm.)	Arteriolar hyalinization	Blood pressure	Disease process
18098	50	434	133	4.6	283	24	CF	600	170	36	+	230/135	Basophilic pituitary adenoma, adrenal hyperplasia
18190	46	420	124	5.6	143	60	WM	480	550	17	+	110/60	Cirrhosis, hepatoma
18338	40	388	118	5.7	68	36	WM	530	410	18.5	0	100/60	Rheumatic heart disease
18163	32	342	132	2.7	236†	47	CM	510	320	28.5	+++ (with necrosis)	220/130	Malignant nephrosclerosis
18107	32	404	127	4.2	59†	43	CF	330	420	14	0	120/85	Viral hepatitis
18148	24	294	121	5.1	55	48	WM	690	446	27	0	100/80	Rheumatic heart disease
18287	20	315	134	3.7	26	65	WF	362	298	11	+	150/90	Carcinoid of ileum with pulmonic stenosis
18319	14	383	132	3.5	38	74	WF	270	320	20	0	130/70	Carcinoma of pancreas
18211	12	300	127	4.4	47	78	WF	330	245	15	+++	150/80	Myocardial infarction
18127	10	292	136	3.6	61	67	WM	350	320	13	+	160/100	Pulmonary emphysema
18292	9	365	125	4.1	19	55	CM	740	480	16	0	110/70	Rheumatic heart disease
18294	8	342	120	4.7	190†	68	CF	313	231	7	+++	180/110	Diabetes, renal abscess
18167	4	311	141	4.1	62	73	WF	360	160‡	11.5	++	146/56	Carcinoma of kidney
18368	4	373	136	3.7	8	59	WF	434	365	13	++	100/82	Carcinoma of breast
18114	2	243	155	3.0	38	39	WF	350	300	22	+	150/90	Glioblastoma multiforme
18193	2	316	135	4.5	50	62	WM	No permit	310	20.5	+++	200/135	Arteriolar nephrosclerosis, cerebrovascular accident
18217	2	310	146	3.9	75	49	WF	610	330	15	++	140/98	Rheumatic heart disease
18112	0	254	136	4.4	19	49	CF	680	250	18	++	120/95	Cirrhosis
18129	0	116	144	3.9	18	1.1	CF	66	123	10	0		Lung abscess
18145	0	363	140	4.5	50	60	WM	500	300	26	+++	170/100	Parathyroid adenoma
18182	0	355	134	7.0	227†	47	WF	250	450	11	+	160/94	Hypoxic nephrosis
18250	0	321	140	3.9	83	50	WF	400	250	20	+++ (with necrosis)	140/90	Malignant nephrosclerosis
18312	0	358	132	4.9	335	19	WF	290	520	14	0	150/70	Hypoxic nephrosis
18348	0	284	135	3.2	317	36	CF	No permit	162	28	+++ (with necrosis)	180/110	Malignant nephrosclerosis

* JGI indicates juxtaglomerular granulation index.

† Symbol indicates blood urea nitrogen; no symbol indicates nonprotein nitrogen.

‡ Right kidney only; left kidney resected.

determinations within the last week of life did not give an adequate representation of the levels of plasma electrolytes for correlation with cytologic findings at death. Some of the observations on the remainder of the 200 cases, however, are also included here. The average was taken of levels of plasma sodium, potassium, and nonprotein nitrogen determined during the last week of life. In some cases where the nonprotein nitrogen was unavailable, the level of blood urea nitrogen was substituted.

Adrenal glands were examined from the selected group of 24 cases. The reticulum pattern proved the most satisfactory method for defining the limits of the cortical zones.[12] Frequently the boundary between zona glomerulosa and zona fasciculata was extremely irregular and difficult to determine. Consequently, the thickness of the zona glomerulosa was measured at 20 to 30 randomly selected sites and an average taken. All measurements and counts were done without the observer knowing the clinical data from the case.

Weights of heart, kidneys, and adrenals were noted at necropsy, and the degree of hyalinization of the preglomerular afferent arteriole was recorded as none, slight, moderate, or advanced (0, +, ++, +++). The statistical relationships between these elements were evaluated. Data from this group of cases are found in Table I.

Results

In the entire series of 200 cases, the degree of granulation of the juxtaglomerular apparatus was less in children under one month of age than in older children or adults. (Seven of 12 had no detectable granules, and the remainder only a few.) No elevated JGI was noted before one month of age, and only one instance occurred in the one-month to one-year group. There was great overlap in the range of values found in the remainder of the age groups. Because of the skewness of the distribution of values of JGI (a few values were greatly elevated, but most were less than 10), the median rather than the arithmetic mean perhaps gives a better picture of the normal. Although the average JGI for males (10.9) was higher than that for females (5.6), and the average for whites (9.0) higher than that for colored (6.9), the median values were not significantly different (males 4, females 2, white 2, colored 2), and there were great overlaps in the range of values. Consequently, significance could not be assigned to these factors in this series. See Table II for a summary of characteristics of the series as a whole (200 cases).

In the smaller group of 24 cases defined above, the JGI correlated

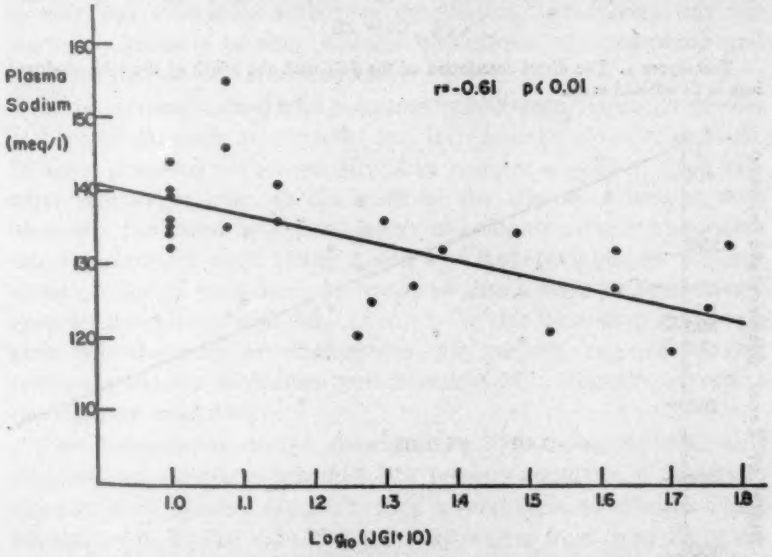
inversely with the level of plasma sodium (Text-fig. 1) and directly with the width of the zona glomerulosa (Text-fig. 2). The $\log_{10}$ (JGI + 10) was used for correlation in order to obtain linearity.[18] In the group of cases with 0 or 1+ degrees of hyalinization of the preglomerular arteriole, the JGI was significantly higher than in those with

TABLE II

Summary of Characteristics of 200 Cases

Number of cases					Age		JGI		
Males	Females	White	Colored	Total	Average	Range	Average	Median	Range
110	90	159	41	200	45.6	0–86	8.6	2	0–120

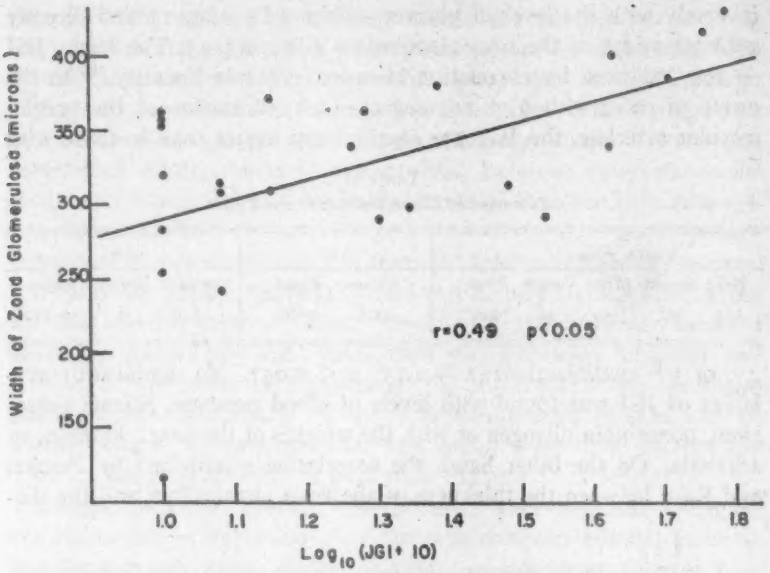
2+ or 3+ hyalinization. ($t = 2.15$; $p < 0.05$). No significant correlation of JGI was found with levels of blood pressure, plasma potassium, nonprotein nitrogen or with the weights of the heart, kidneys, or adrenals. On the other hand, the correlation established by Peschel and Race between the thickness of the zona glomerulosa and the die-



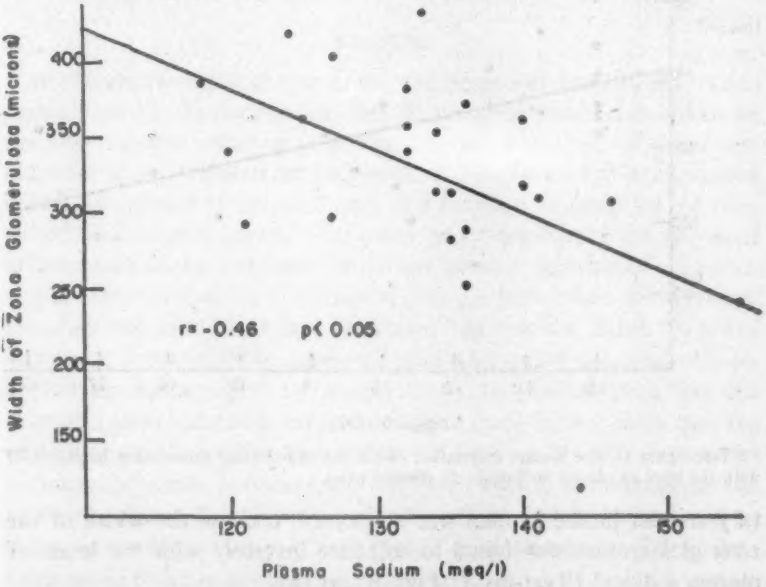
Text-figure 1. The inverse correlation of the juxtaglomerular granulation index (JGI) with the level of plasma sodium in 24 selected cases.

tary sodium intake in man was supported, because the width of the zona glomerulosa was found to correlate inversely with the levels of plasma sodium[5] (Text-fig. 3; Figs. 9 and 10).

The anatomic changes of the juxtaglomerular cells with hyper-



Text-figure 2. The direct correlation of the JGI with the width of the zona glomerulosa in 24 selected cases.



Text-figure 3. The inverse correlation of the width of the zona glomerulosa with the level of plasma sodium in 24 selected cases.

granulation were fairly consistent. When only a few granules were present in the cell, they were usually found in perinuclear sites, although they could be found anywhere in the cytoplasm (Fig. 1). Granulation could be of sufficient amount, however, to fill the cell completely (Fig. 3). Such degrees of granulation were unusual in these studies on man. Granules were, in general, more difficult to find in human kidneys than in those of most laboratory animals. In view of the very low JGI normally found in man, degranulation could not be evaluated as readily as in animals. In the rare instances of extreme hypergranulation, the granular cells extended proximally from the glomerulus nearly down to the origin of the arteriole (Fig. 4). Those surrounding the preglomerular arteriole increased in number. The wall of the arteriole became thicker in an eccentric manner and appeared to bulge into the lumen of the tubule (Figs. 5 and 6). The thicker side lay next to the *macula densa*. Intermingled with the granular cells, cells with clear agranular and afibrillar cytoplasm could be seen (Figs. 1 and 4). Although hyperplasia of the juxtaglomerular elements usually was associated with hypergranulation, hyperplasia was not studied sufficiently to state whether dissociation of hyperplasia and hypergranulation occurred frequently.

In the sections stained with hematoxylin and eosin, advanced degrees of hyperplasia could be detected and increased granulation assumed. If many glomeruli with unusually large nodules of cells at their vascular poles were seen, or the walls of the afferent arterioles were unusually thickened by several layers of cells, then hypergranulation was frequently present (Figs. 1 and 2). Hypergranulation was not always found in such cases, however, so that a stain to demonstrate granules was always necessary (Fig. 7). In this laboratory the Bowie stain was the most satisfactory for this purpose, but the Masson trichrome and the McManus periodic acid-Schiff stains also served to demonstrate granularity.

The juxtaposition of the *macula densa* and juxtaglomerular cells suggested an associated function. The primary emphasis of this study was not on the *macula densa* but upon several types of changes which were noted in it. The *macula densa* cells ranged from cuboidal to columnar in height (Figs. 5 and 6). In some cases the ratio of the number of *maculae densae* to glomeruli was increased over normal, indicating an increase of the former. Basilar vacuolization was noted frequently (Figs. 5 and 6). In some instances in which tubular degeneration was observed, the *macula densa* appeared relatively spared. No correlation of these changes with the clinical factors studied was demonstrated.

Discussion

The specialized elements in the walls of preglomerular renal arterioles referred to as the juxtaglomerular apparatus were first noted by Ruyter[14] in 1925 although it was not until 1927 that their existence in the human kidney was established.[15] Goormaghtigh pioneered the attempt to define the function of this group of cells. He felt that they had an endocrine function and postulated that they were the source of the vasopressor, renin.[16,17] Hypergranulation of the juxtaglomerular cells has been shown to occur during the early stages of experimental renal ischemia[18] but has not been noted to accompany benign hypertension in man. The early work in this field was concerned primarily with the phenomenon of hyperplasia rather than hypergranulation.

Variation of granulation in the juxtaglomerular apparatus with the level of plasma sodium further suggests an endocrine function. The behavior of the granulation, if hypergranulation can be equated with increased function, is that which would be expected if the granules represented a trophic hormone or its precursor. In response to a low level of plasma sodium, hypertrophy of the zona glomerulosa and increased secretion of mineralo-corticoids would be anticipated. This concept is also compatible with the observation of Dunihue (supported by a case in this series of primary atrophy of the adrenals) that hypergranulation is found in adrenalectomized animals.[4,5] However, these data are not in accord with the conception suggested by Dunihue and Robertson[19] that granulation is correlated inversely with the blood level of mineralo-corticoids.

Juxtaglomerular cells have been proposed as the source of renin.[16,17] The data presented here are compatible with this hypothesis if it is assumed that the renin-hypertensin system is essentially a homeostatic mechanism analagous to the carotid sinus reflex. A fall in blood pressure would call forth a renin response, and a rise would decrease the output and be reflected by the presence of fewer granules in the juxtaglomerular apparatus (low JGI). Other evidence, however, suggests that renin is found primarily in the proximal convoluted tubules.[20]

The possibility that the juxtaglomerular apparatus produces a hormone which has combined vasopressor and sodium retaining properties should be considered. Renin extracts have been shown to increase the thickness of the zona glomerulosa, and thus appear to have a trophic as well as a vasoconstrictor effect.[21] The relationship of blood pressure and sodium metabolism is so intimate, however, that their regulating mechanisms may well be combined at times.

Although in this group of cases the level of nonprotein nitrogen

(NPN) did not correlate significantly with the JGI, many of the cases with an elevated JGI were noted also to have an NPN which was elevated to some degree. However, in the absence of a low plasma sodium level, an elevated JGI was not found even with a greatly elevated NPN. A high level of plasma potassium, although not significant in itself, seemed in some cases to enhance the effect on the JGI of low plasma sodium levels. Some of the cases with the highest JGI had, in addition to low plasma sodium levels, evidences of hyperkalemia. The specific nature of the disease process did not appear important with respect to the elevation of JGI. Indeed, as indicated by McManus,[22] cirrhosis, hypoxic nephrosis, malignant hypertension, and Addison's disease were frequently noted to be associated with hypergranulation of the juxtaglomerular cells. A number of these patients also had low plasma sodium levels at death, but in those cases in which the plasma sodium was normal terminally, no hypergranulation was found.

The height of the blood pressure, which has frequently been related to the juxtaglomerular apparatus, was not found to be correlated significantly with the JGI in this group. The tendency, if any, appeared to be an inverse one. In animals an inverse relationship of this nature has been established.[23] Perhaps a larger group of cases would show significant correlation, but if this were so, it would probably be readily obscured by any associated changes in levels of plasma sodium. The definite inverse correlation of JGI with preglomerular arteriolar hyalinization is somewhat surprising in view of this observation and suggests that further studies should be done.

It is tempting to try to associate the sparsity of granules in the juxtaglomerular apparatus in the kidney of the very young with the known immaturity of renal function in the infant. However, this feature may only be indicative of the nature of the disease and the rapidity with which infants die.

The relationship demonstrated between the JGI, the width of the zona glomerulosa of the adrenal cortex, and the plasma sodium level extends the earlier work carried out in animals to man.

Summary

Sections of kidneys procured from an unselected group of 200 patients at necropsy were stained to demonstrate granules in the juxtaglomerular apparatus. A sub-group of 24 cases was selected on the basis of the availability of adequate studies of plasma sodium levels during the last week of life. Three determinations during this period were deemed sufficient for this purpose.

The degree of granulation of the juxtaglomerular apparatus was found to be correlated inversely with levels of plasma sodium and directly with the width of the zona glomerulosa of the adrenal cortex. Other factors considered in the study were thought to play subsidiary roles in this association. An inverse relationship between the levels of plasma sodium and the thickness of the zona glomerulosa has been demonstrated in animals; this study shows that the relationship holds true in man, as well.

The possible functions of the juxtaglomerular cells are discussed.

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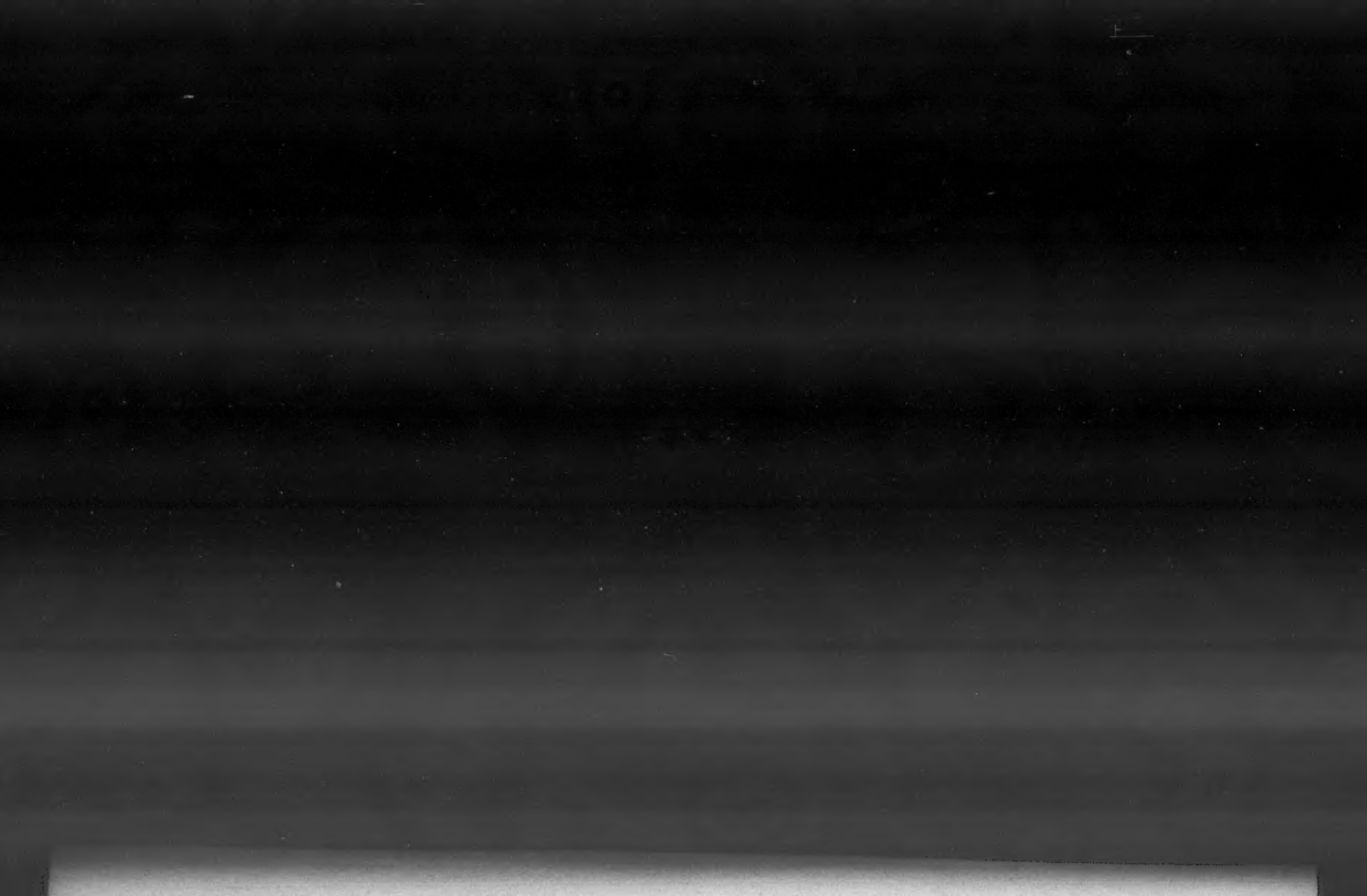
We wish to acknowledge many helpful suggestions and criticisms from Professor W. S. Hartroft and Professor Margaret G. Smith.

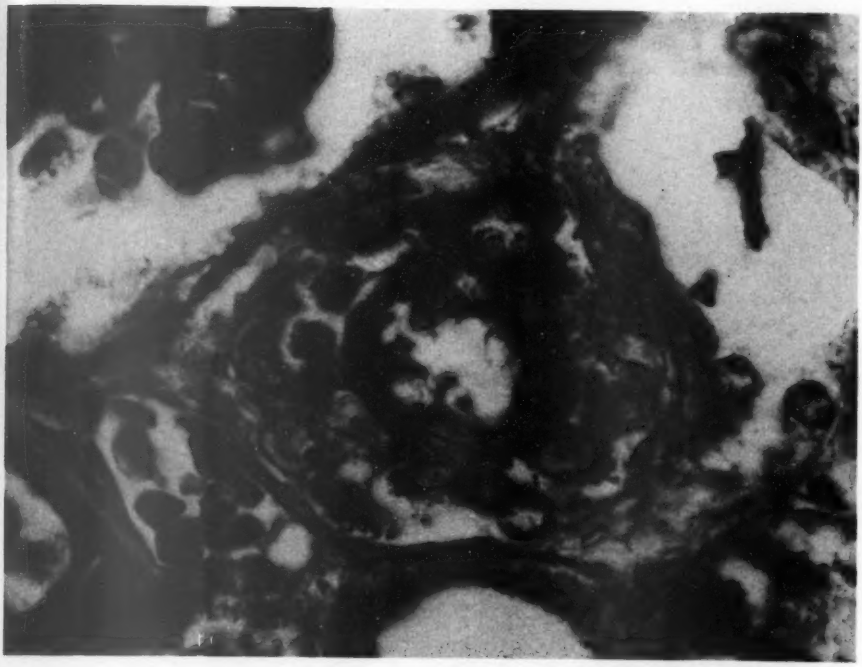
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Case 18133. Cross section of an afferent arteriole adjacent to a glomerulus. Granular and agranular juxtaglomerular cells cause thickening of its wall (JGI = 104). Note the perinuclear distribution of granules when only a few are present. Bowie stain. × 1,200.

FIG. 2. Case 18133. Cross section of an afferent arteriole with cellular thickening of wall. Hematoxylin and eosin stain, to be compared with Figure 1. × 1,200.





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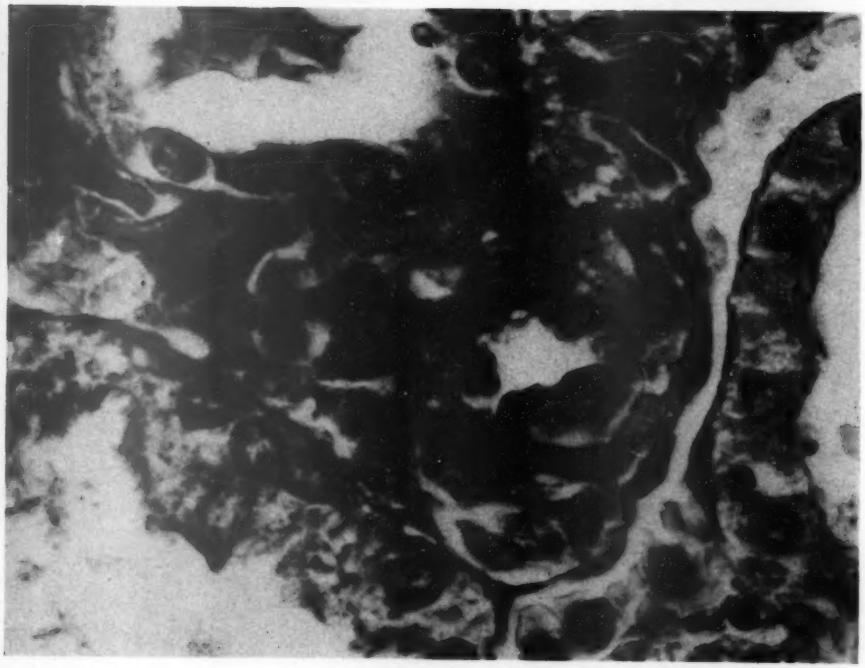

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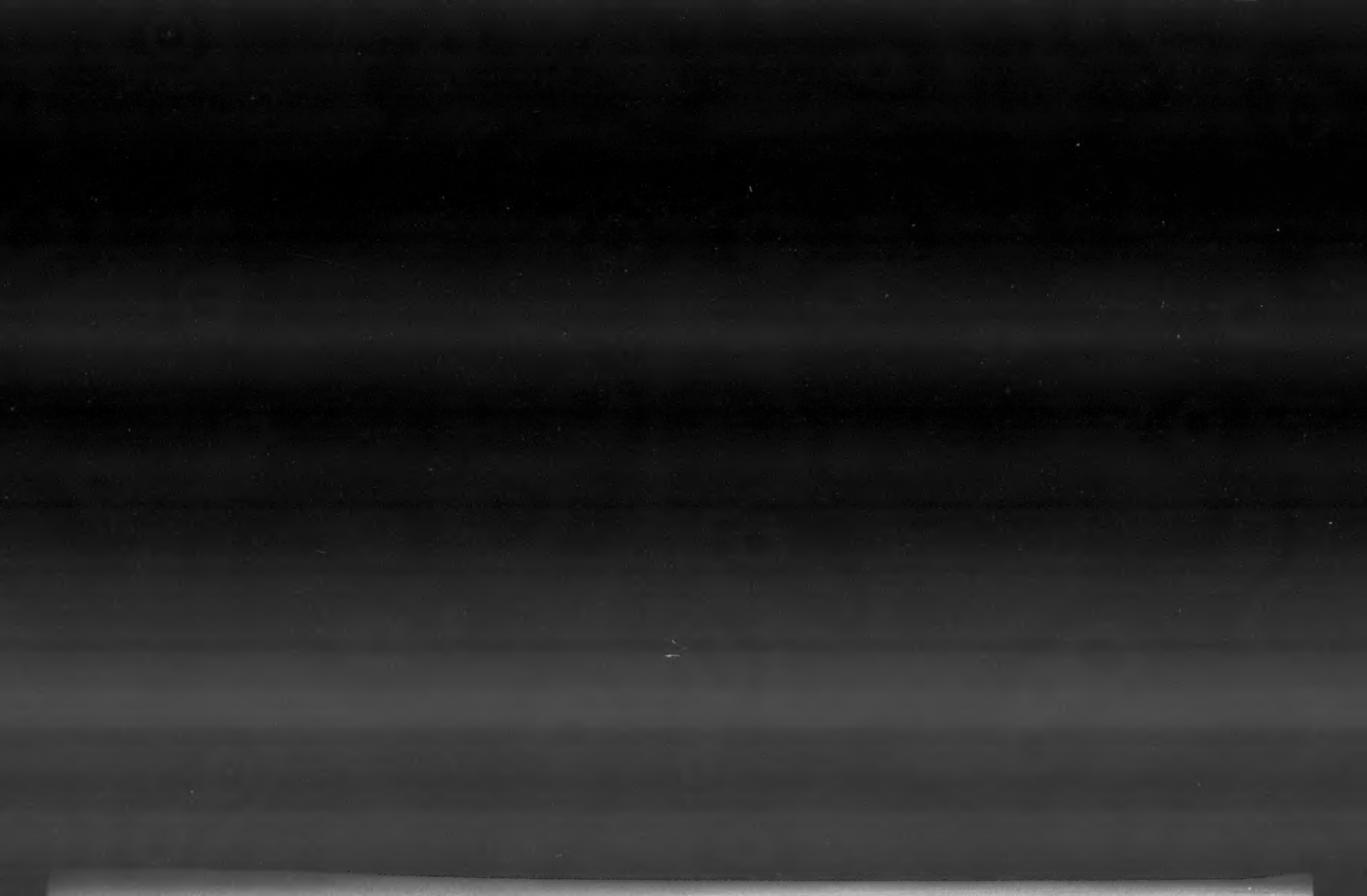
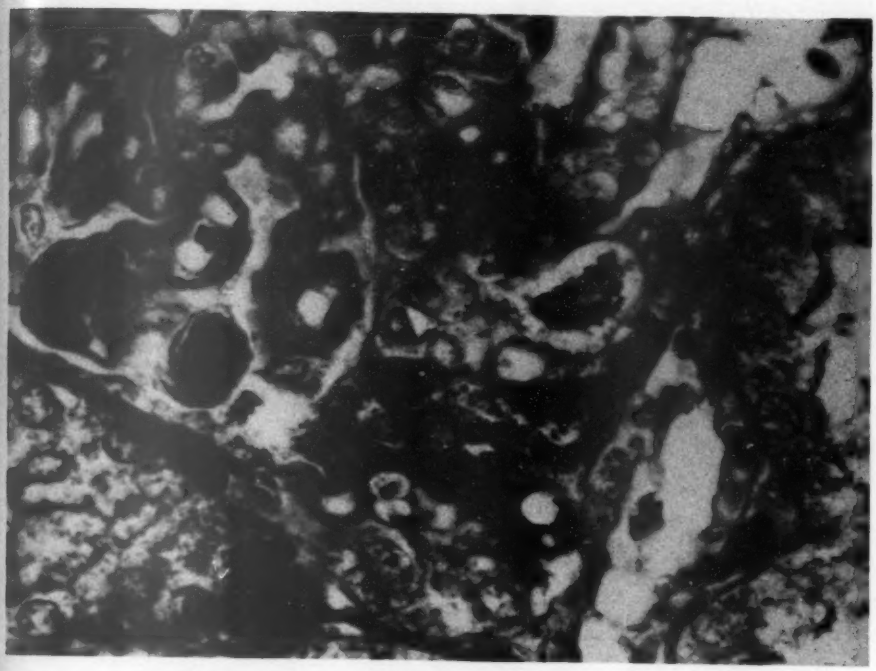
FIG. 3. Case 18133. Section through a glomerular root, demonstrating advanced hypergranulation of the juxtaglomerular cells. (JGI = 104). Bowie stain. × 1,000.

FIG. 4. Case 18133. Section of an afferent arteriole proximal to a glomerulus, showing granular cells extending down an arteriole. The glomerulus is above the field shown. (JGI = 104). Bowie stain. × 1,000.





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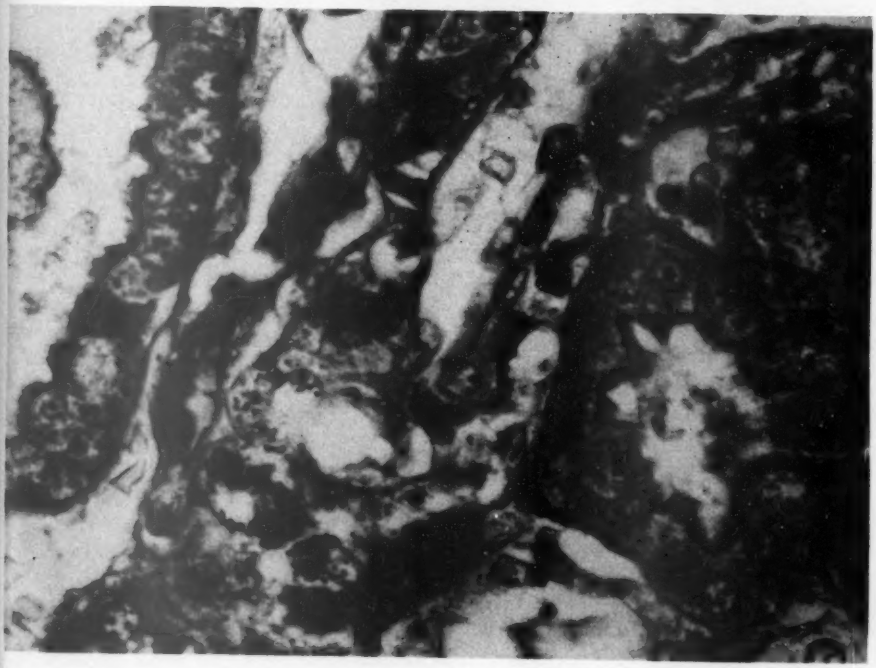

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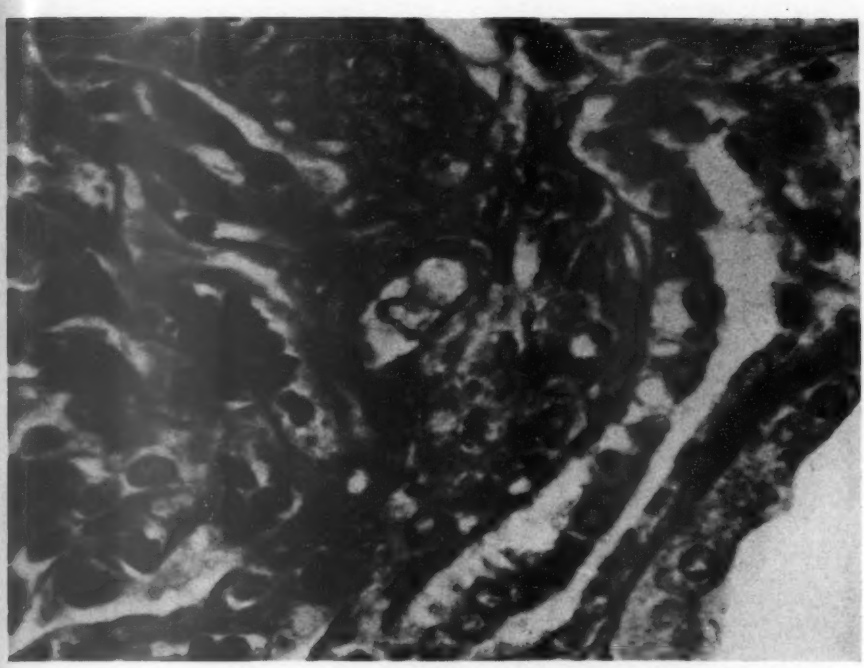
FIG. 5. Case 18338. Section of a glomerular root. Eccentric hyperplasia of juxtaglomerular cells causes bulging into the lumen of the distal tubule (JGI = 40). Note basilar vacuolation of *macula densa* cells. Bowie stain. × 1,000.

FIG. 6. Case 18338. Section at a different angle from that shown in Figure 5, demonstrating eccentric hyperplasia of the juxtaglomerular cells. Hematoxylin and eosin stain. × 1,000.



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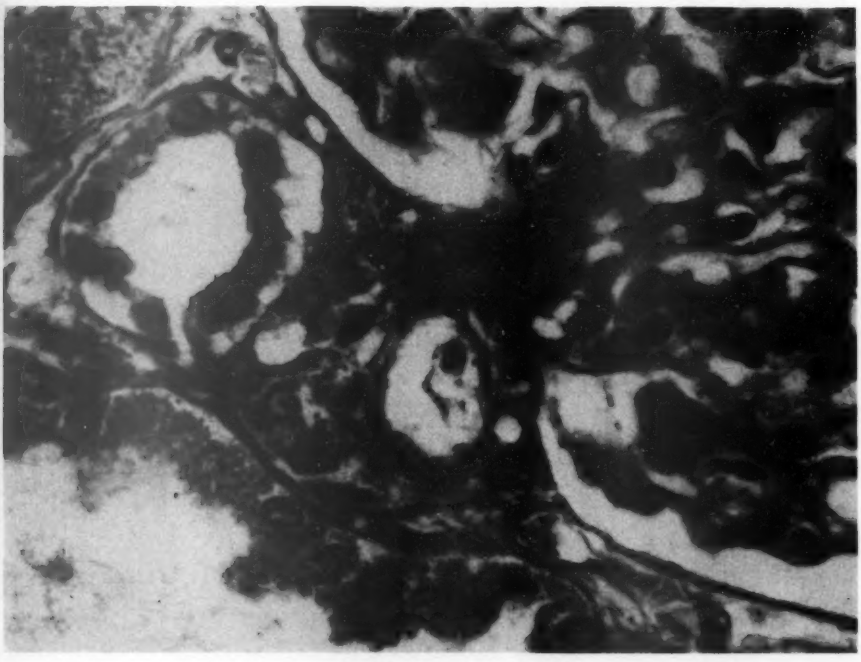

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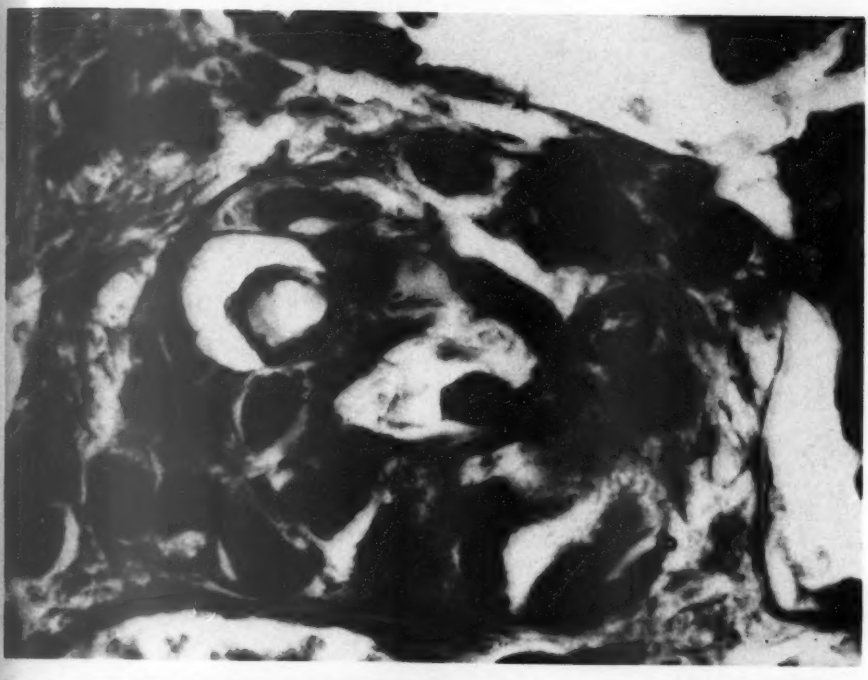
FIG. 7. Case 18217. Cross section of an afferent arteriole with apparent thickening of its wall. In a section stained with Bowie's stain increased granulation was not shown (JGI = 2). Hematoxylin and eosin stain. × 1,900.

FIG. 8. Case 18133. Section through an afferent arteriole and *macula densa* with eccentric hyperplasia of juxtaglomerular cells. Hematoxylin and eosin stain. × 1,000.



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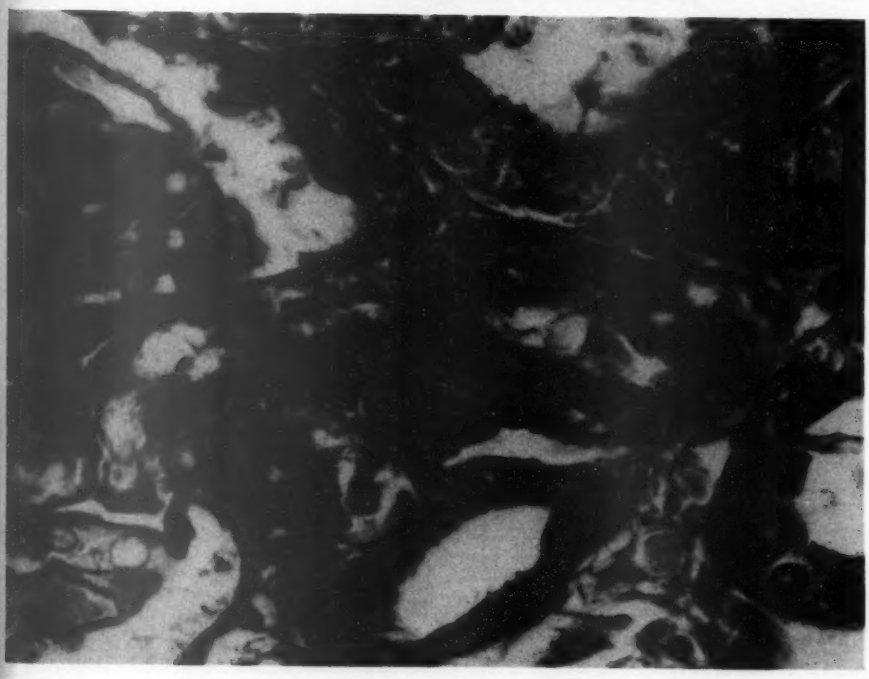

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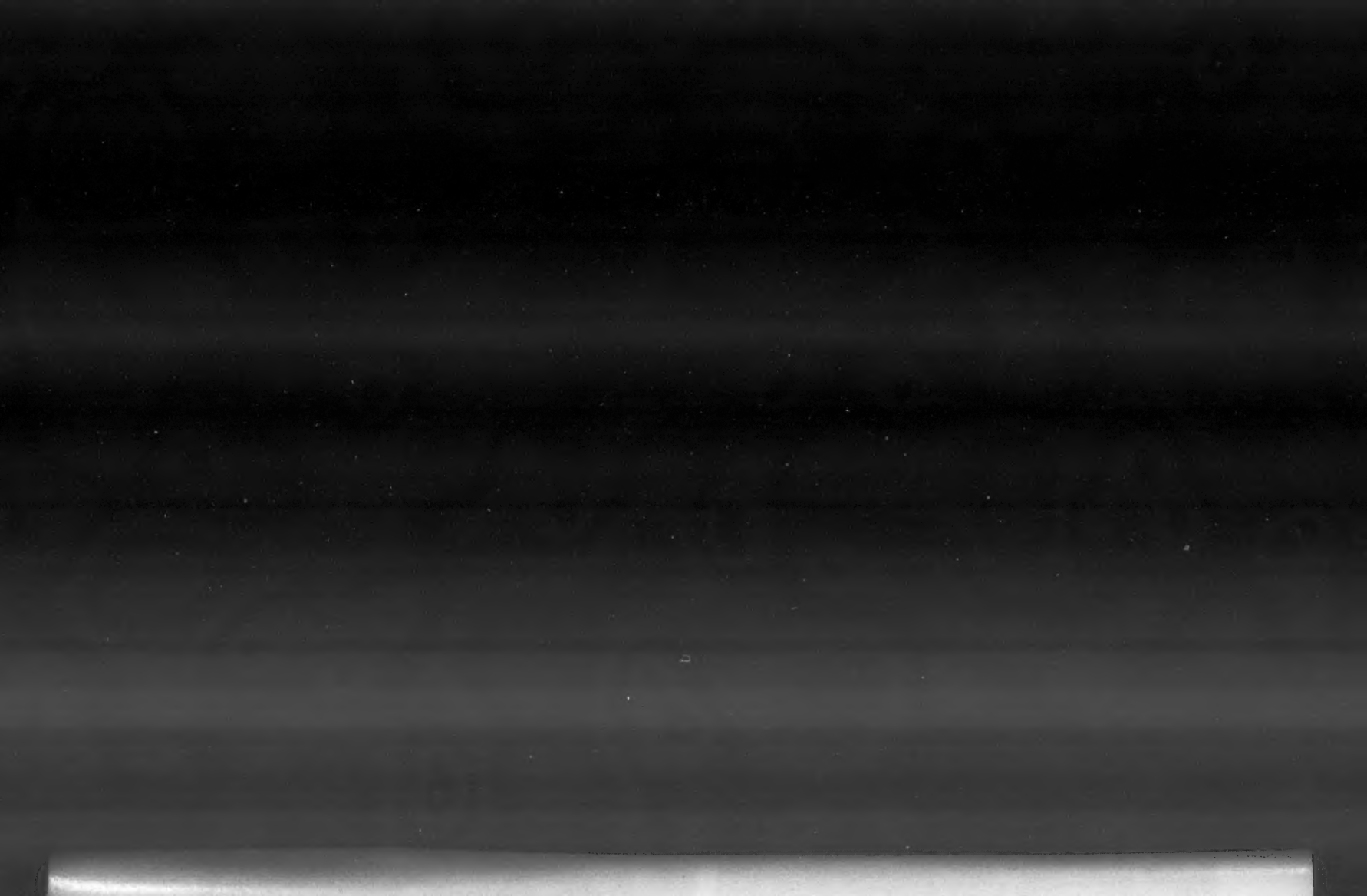
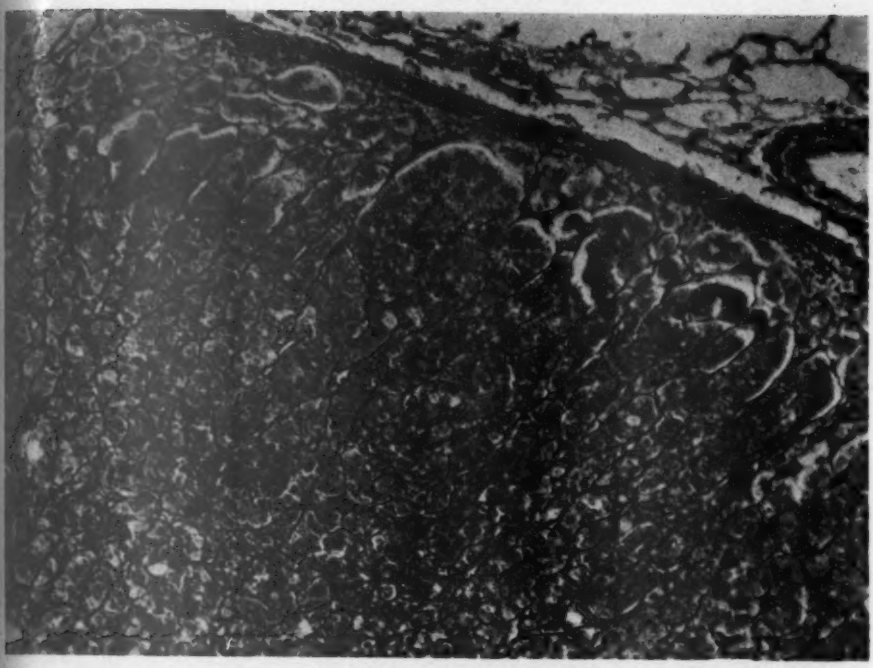
FIG. 9. Case 18114. Adrenal cortex from a case with normal plasma sodium level. Boundary between reticulum patterns of zona glomerulosa and zona fasciculata is irregular. Wilder reticulum stain. × 100.

FIG. 10. Case 18107. Adrenal cortex from a case with low plasma sodium level. Widening of the zona glomerulosa is apparent although the boundary with the zona fasciculata is irregular. Wilder reticulum stain. × 100.





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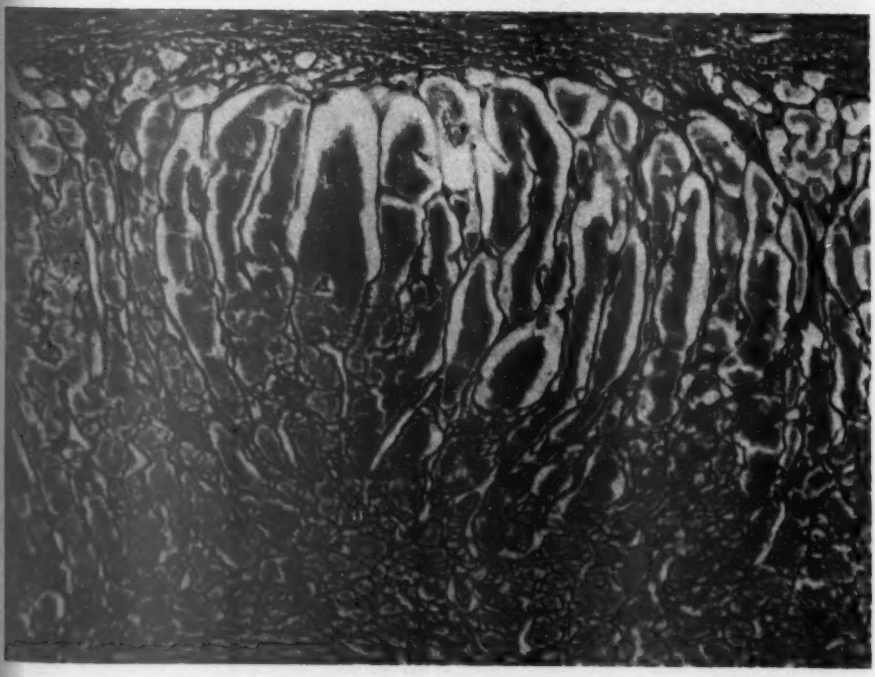

10

ELECTRON MICROSCOPY OF THE MAMMALIAN RENAL GLOMERULUS

The Problems of Intercapillary Tissue and the Capillary Loop Basement Membrane *

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The presence and nature of intercapillary tissue in the renal glomerulus have been controversial points for many years. Greenfield,[1] Herring,[2] Kimmelstiel and Wilson,[3,4] Zimmerman,[5] Ehrich,[6] Bensley and Bensley,[7] and Goormaghtigh[8] considered that it did exist and was probably fibrous tissue in nature. McManus[9] and Yamada[10] considered that a third type of tissue was present in the glomerulus but could not stipulate its exact nature. Bell,[11] Allen,[12] Pease and Baker,[13] Rinehart, Farquhar, Jung and Abul-Haj,[14] Farquhar, Vernier and Good[15] denied its existence, while Hall,[16] Mueller, Mason and Stout,[17] and Bergstrand[18] claimed that although cells were present, they represented the endothelium of capillaries in other planes. It appears that the majority of workers agree that there is some tissue present, which, in the plane of sectioning, is exposed neither to blood nor to Bowman's space.

Materials and Methods

Glomeruli of normal rats' kidneys and a human kidney were prepared for electron microscopy as described in previous papers,[19-21] but the fixation time was reduced to one hour. The cortical wedge of human kidney was removed from a male, aged 76, in whom a nephrectomy was performed because of a large calculus. The sections were examined in a Philips electron microscope, type E.M. 100 at 60 k.v., using a 50 μ objective aperture.

Results

There was little doubt that in rats and the human subject, there was, in the plane of sectioning, a mass of tissue composed of cells with nuclei, projections of cytoplasm, and dense intercellular material. This was separated from the capillary lumen by endothelial cytoplasm and from Bowman's space by a branch of the capillary loop basement membrane, on which rested epithelial pedicels (Fig. 1). There were no distinguishing features in the cytoplasm or nuclei of these cells. The substance is designated as intercapillary tissue in the rest of this paper, since this terminology is noncommittal as to its nature.

At the junction between the intercapillary tissue and the capillary

* Received for publication, February 28, 1958.

lumen, the endothelium was separated from the cells of the former by either mottled intercellular material or a dense layer similar to that of the capillary loop basement membrane. This dense layer was termed the subendothelial basement membrane (Fig. 10). When the main body of endothelial cytoplasm (and the nucleus) was situated at this junction, it generally projected into the lumen, but occasionally extended into the intercapillary tissue (Fig. 3). A section through this structure, at right angles, would exhibit the projection of endothelial cytoplasm in the center of the intercapillary tissue. The frequency with which intercapillary tissue was observed in sections and the infrequency of endothelial projections make it unlikely that all the intercapillary tissue is of endothelial nature.

In the middle of what appears as intercapillary tissue to the light microscopist, obvious epithelial pedicels were present in electron micrographs (Fig. 4). These are beyond the resolving power of the light microscope. Pedicels in this position were the result of deep penetration from the surface into the intercapillary tissue (Fig. 5), and were recognized by the presence of the outer, less dense layer and Bowman's space. Only rarely were both of these features absent, and the epithelial character was then beyond recognition (Fig. 6). It would appear unlikely, therefore, that much of the intercapillary tissue was of epithelial nature.

The intercellular material of the intercapillary tissue had the same density as the dense layer of the capillary loop basement membrane and was continuous with it. On occasion there were large amounts of this material (Fig. 7). There was a tendency for the intercellular substance to have a mottled appearance in rats (Figs. 4 and 5), and a homogeneous appearance in man (Fig. 7). Although generally intercapillary tissue did not extend into the thin peripheral wall of the capillary loop, occasionally it did so (Fig. 2). When this happened, the dense layer of the capillary loop basement membrane remained on the side of Bowman's capsule, retaining its relationship to the epithelial pedicels (Fig. 2). The endothelium bore the same relationship to this extension as to the main body of intercapillary tissue. This extension has been designated pericapillary tissue.

Since Remak[22] published his paper on the invagination theory in the formation of the renal glomerulus, many authors have concluded that there must be two components to the capillary loop basement membrane which become visible in disease processes: a subendothelial and a subepithelial portion. Electron microscopic studies have not so far confirmed these conclusions. In the present study in rats, the base-

ment membrane at the periphery of the capillary loop was composed of the 3 layers previously described (the outer less dense layer, middle dense layer, inner less dense layer)[10,18,19,23-25] (Fig. 8). The outer less dense layer was a fairly constant feature and was only occasionally absent. This had also been observed previously by Farquhar and co-workers.[15] The inner less dense layer was absent much more frequently. The inner margin of the dense middle layer occasionally had a mottled appearance, and the dense layer was broken into several thin layers in a manner similar to that described in the marsupial renal glomerulus.[19] In the human glomerulus the 3 layers were frequently not seen. The outer less dense layer was usually absent, the inner less dense layer was present only occasionally, and the dense middle layer was wider. The dense middle layer was regarded as the basement membrane proper.[16] At the junction of the capillary loops and intercapillary tissue, when there was a definite subendothelial basement membrane, it joined the dense layer surrounding the intercapillary tissue to form the basement membrane of the capillary loop. Occasionally, some of the less dense, mottled intercellular material separated these two layers for a short distance into the loop (Fig. 10). This separation might continue into the periphery of the loop (Fig. 11), but usually, as previously stated, the basement membrane comprised a dense homogeneous layer. Farquhar and co-workers[15] have described a similar alteration of the basement membrane in cases of familial nephrosis. When the endothelium was separated from the intercapillary tissue by mottled intercellular material, no definite union with the dense layer surrounding the intercapillary tissue was seen.

At the hilus, where the adventitial coat of the arteriole penetrated the glomerulus, Bowman's capsular basement membrane joined the intercellular material. A dense layer of this material separated the adventitial cells from the epithelial pedicels in exactly the same manner as intercapillary tissue was separated from the epithelial pedicels. In only one of many sections was the capsular basement membrane seen to join the dense layer beneath the arteriolar endothelium to form the dense layer of a capillary wall.

Discussion

The intercapillary tissue observed with the light microscope is found by electron microscopy to be composed of intercellular material, endothelial cells, epithelial cells, and a third type of cell, the nature of which could not be established. However, since the intercapillary

tissue and the adventitial coat of the arteriole bear the same relationship to the epithelial pedicels, it appears that this third type of cell may be of fibroblastic nature. The continuation of the central cell mass into the adventitial coat of the arteriole in the avian glomerulus[25] further supports this concept. Similar masses of intercapillary tissue have been described in the plane of sectioning in marsupial,[19] avian,[25] and reptilian (unpublished data) metanephric glomeruli, and in the amphibian[21] and piscine[20] mesonephric glomeruli.

The observation of a subendothelial dense layer at the junction of the capillary loop and intercapillary tissue, although an infrequent occurrence, and its union with the dense layer surrounding the intercapillary tissue, supports the observations in diseased glomeruli with the light microscope by Jones,[26] and by Grishman and Churg.[27] The occasional occurrence of two dense layers (subepithelial and subendothelial) in normal glomeruli, with a mottled intercellular substance between them in the free wall of the capillaries, suggests that the basement membrane proper is composed of these two components which generally fuse together indistinguishably.

Summary

Electron microscopic study of the glomerulus in rats and human subjects indicated that the intercapillary tissue as observed with the light microscope was composed of intercellular material, endothelial and epithelial cells, and a third type of cell. No direct evidence could be found to determine the nature of this third type of cell although indirect evidence indicated that it was of the same nature as the adventitial cells of the arteriole. The capillary loop basement membrane was formed by the junction of the dense layer surrounding the intercapillary tissue and the subendothelial basement membrane. The latter was occasionally divided into two layers at the periphery of a loop and contained mottled intercellular material or pericapillary tissue between the layers. Generally, however, the layers were fused to form the basement membrane proper of the loop.

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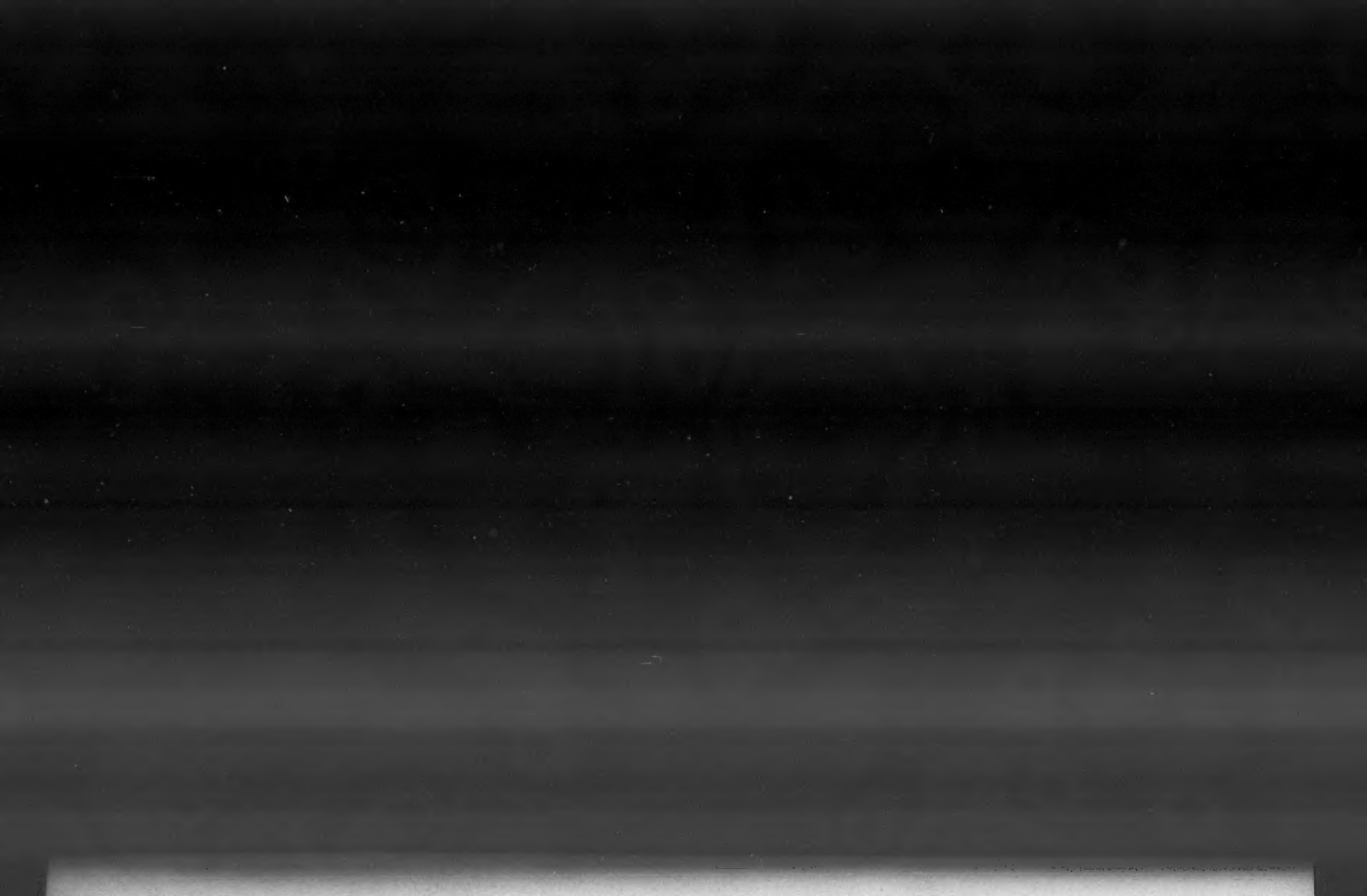
I wish to thank Dr. S. G. Tomlin for access to the electron microscope, Mr. J. Orsula for assistance in its operation, and Professor J. S. Robertson for helpful advice and criticism during this study.

LEGENDS FOR FIGURES

FIG. 1. In the plane of sectioning, intercapillary tissue (W) occupies the center of the electron micrograph, with capillary lumens (C) above and below. Pedicels (P), in Bowman's space, cover the remaining surfaces. Endothelial cytoplasm (E) lines the capillary lumens. × 17,000.

FIG. 2. Intercapillary tissue (W) is seen extending into the peripheral wall of a capillary loop. The capillary lumen (C) is lined by endothelium (ES) and contains a red cell (RC). Pedicels (P) are in Bowman's space and project onto the capillary wall. × 20,000.

FIG. 3. An endothelial cell (E) projects into a mass of intercapillary tissue (W) in the plane of sectioning. A capillary lumen is at (C) and an epithelial pedicel at (P). A section passing through the continuous dark line and at right angles to the present plane would result in this obvious endothelial cell being in the center of intercapillary tissue, and its correct nature would be beyond recognition. × 25,000.



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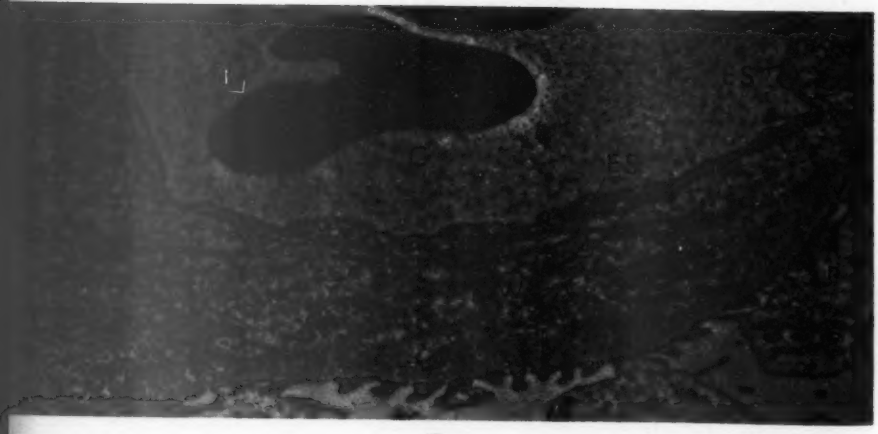

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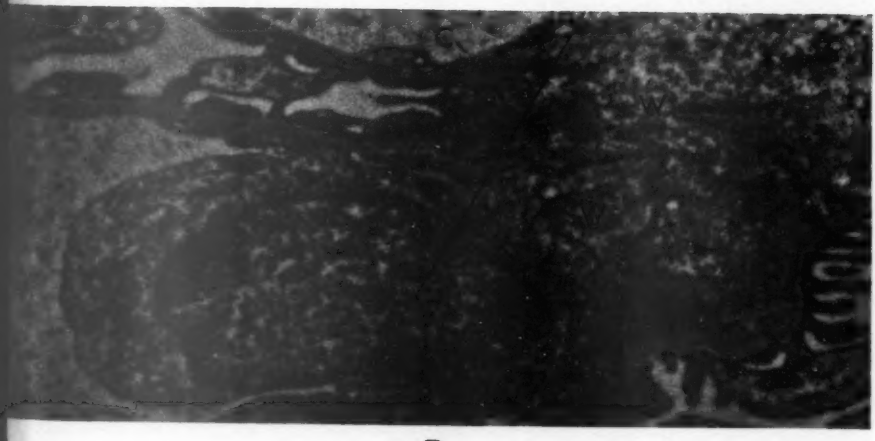

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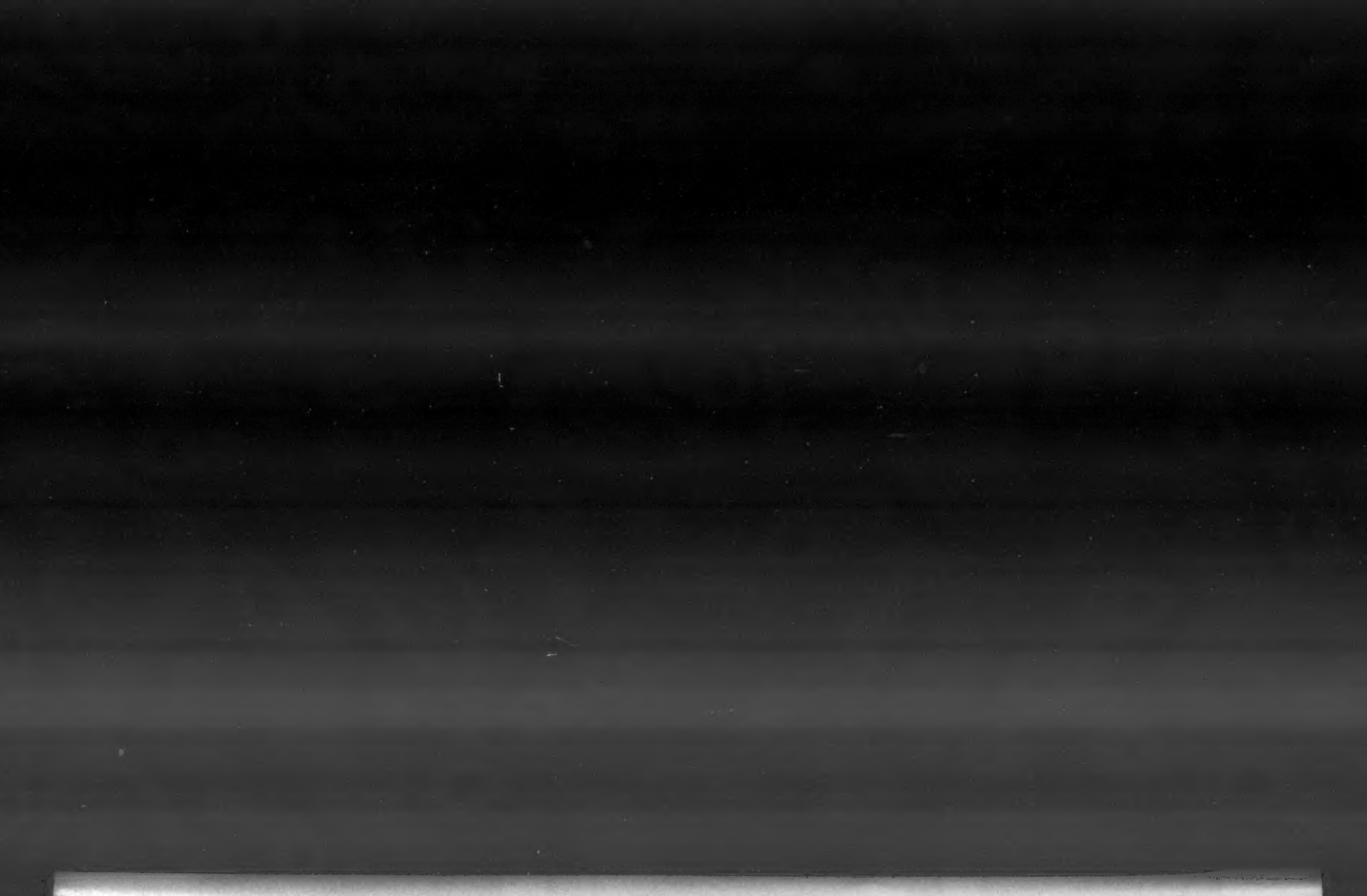
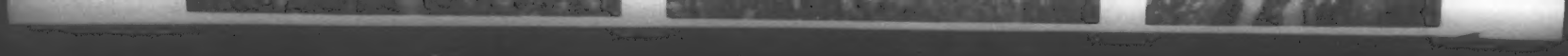
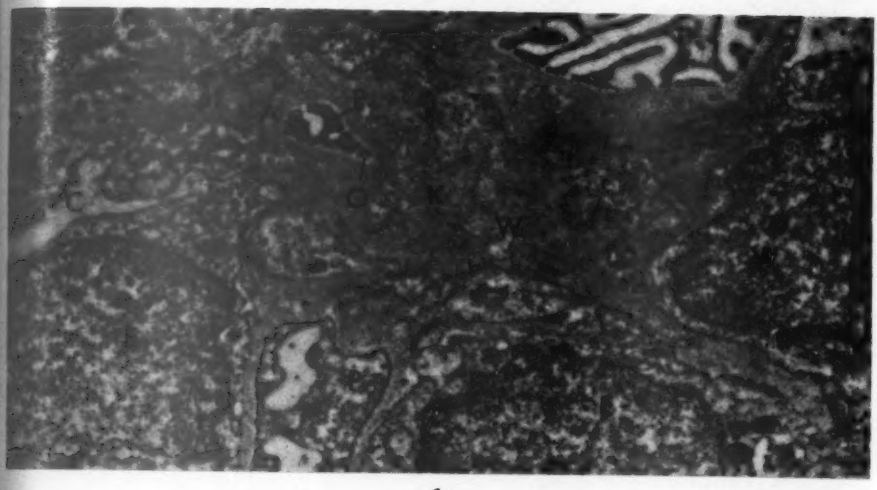
FIG. 4. Slightly to the left of center there are pedicels (P). These, with Bowman's space (A) and the outer less dense layer (O), are present in a mass of tissue (W) which would have been termed intercapillary tissue by the conventional microscopist. The pedicels and the outer less dense layer are beyond the resolving power of the light microscope. There is mottled interceliular material (K) in the intercapillary tissue (W). Pedicels (P) are above and below the intercapillary tissue cells (W). Endothelial cells are present at (E) and a capillary lumen at (C). × 23,000.

FIG. 5. The center of the photograph shows the deep penetration of the outer less dense layer (O), Bowman's space (A) and epithelial pedicels (P) into a mass of tissue which would have been termed intercapillary tissue by the conventional microscopist. Mottled intercellular material of the intercapillary tissue is at (K). Capillary lumens are at (C), an endothelial cell at (E) and pedicels at (P). × 26,000.

FIG. 6. The outer less dense layer of the capillary wall is absent, and a single pedicel (P) penetrates into intercapillary tissue (W). Endothelium (ES) lines the capillary lumen (C). A section through the thin dark continuous line and at right angles to the present plane would result in this pedicel being among a mass of intercapillary tissue and its correct nature beyond recognition. This is an infrequent finding. × 30,000.







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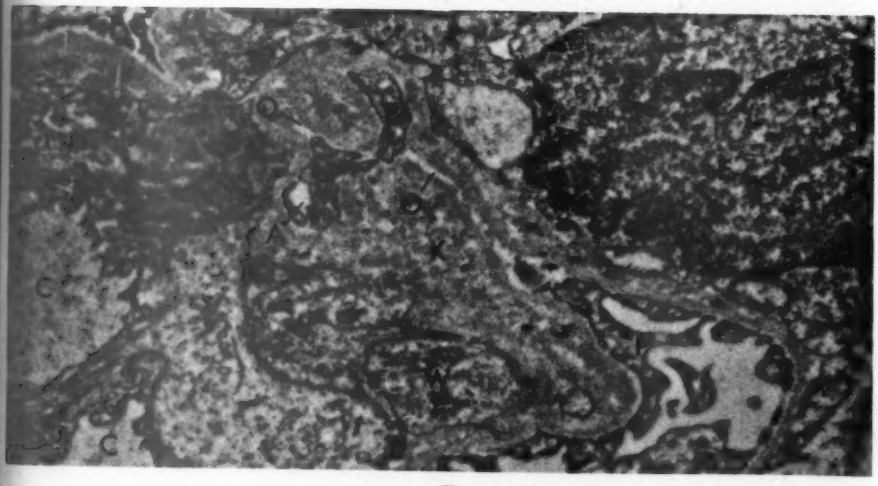

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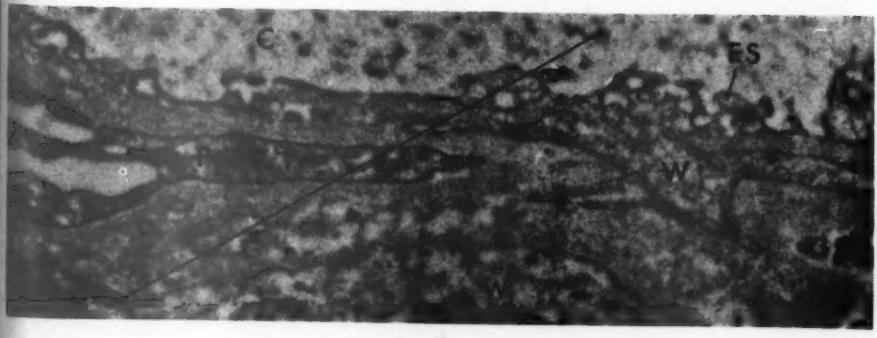

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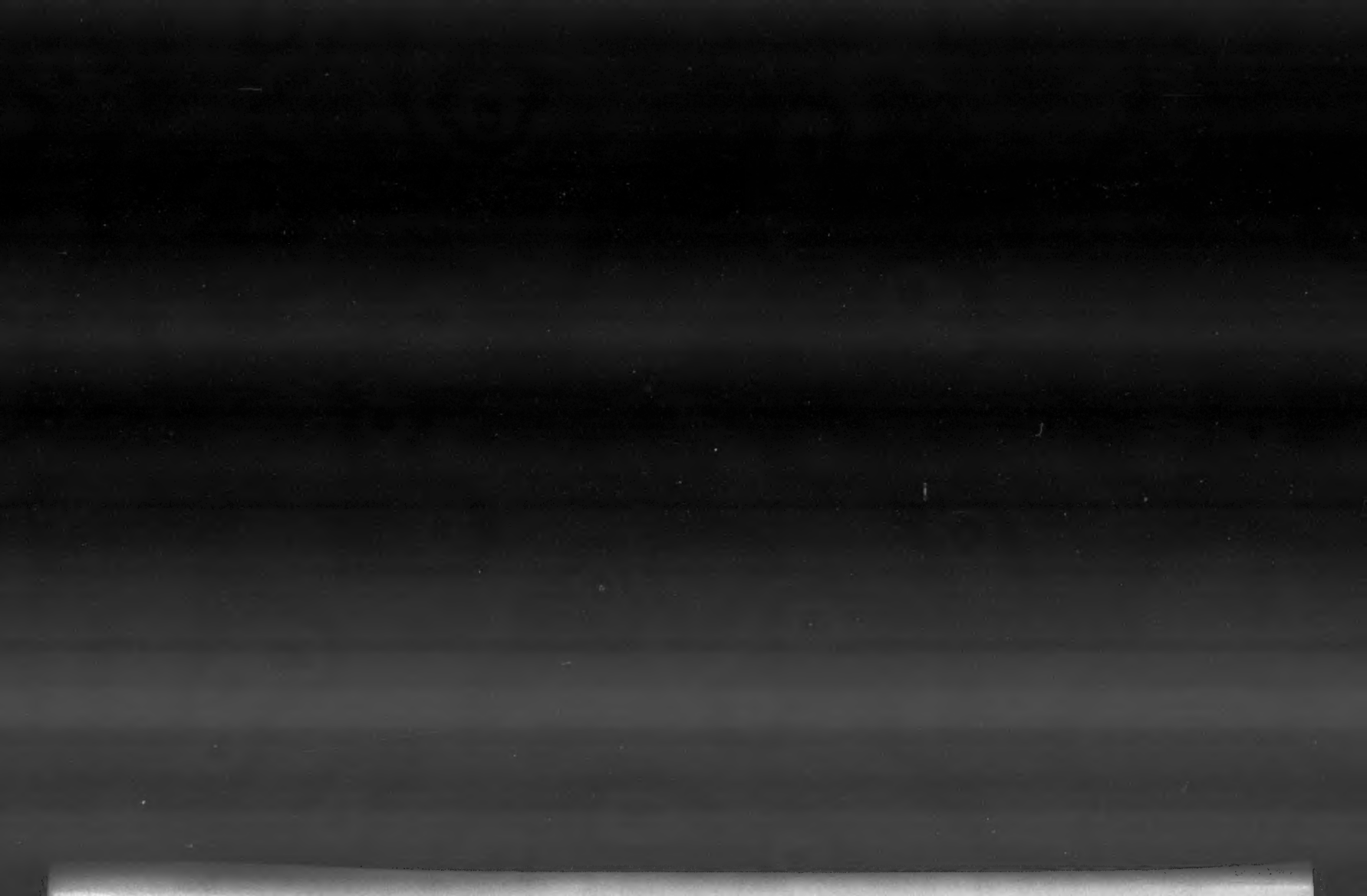
FIG. 7. A section of human kidney, showing the homogeneous intercellular material (K) of the intercapillary tissue (W). An endothelial cell (E) lines the capillary lumen (C). Epithelial pedicels (P) rest on the outer surface of the intercapillary tissue. × 25,000.

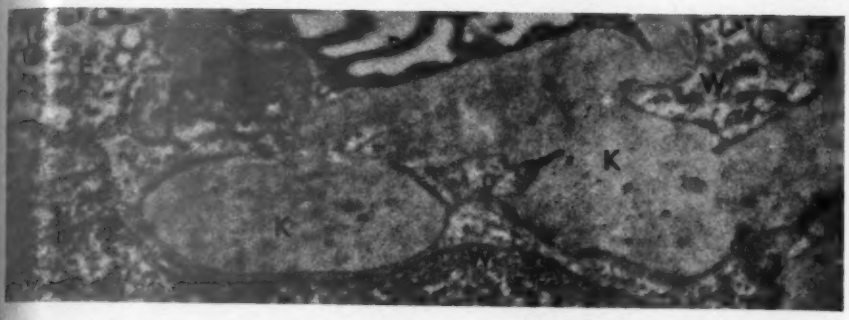
FIG. 8. The peripheral wall of the capillary loop is composed of epithelial pedicels (P), outer less dense layer (O), dense middle layer (D), or basement membrane proper, inner less dense layer (L) and attenuated endothelial sheet (ES). The lumen is at (C). × 55,000.

FIG. 9. The capillary wall of a human glomerulus shows the absence of the outer and inner less dense layers. The middle dense layer (D) comes into contact with pedicels (P) and the endothelial sheet (ES). On the right side, the outer less dense layer (O) can be seen. × 35,000.

FIG. 10. The dense layer (Z) surrounding the intercapillary tissue (W) joins with the subendothelial basement membrane (SB) to form the dense layer (D) of the capillary loop basement membrane. The capillary lumen (C) is lined by endothelium (ES). Pedicels (P) are in Bowman's space. × 35,000.

FIG. 11. There is splitting of the dense middle layer at the periphery of a capillary loop into a dense layer (M) adjacent to the epithelial pedicels (P) and a dense layer (N) beneath the endothelium with mottled intercellular material between these two layers. The capillary lumen is at (C). × 30,000.





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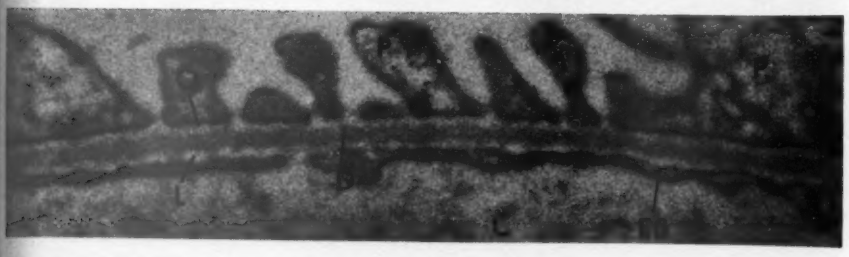

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THE FINE STRUCTURE OF THE AORTIC ENDOTHELIAL LESIONS IN EXPERIMENTAL CHOLESTEROL ATHEROSCLEROSIS OF RABBITS *

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Lesions of arteries induced in the rabbit by feeding a high cholesterol diet have been the subject of many investigations from the time of their discovery by Anitschkow and Chalatow.[1] An excellent review of the subject was given by Duff and McMillan.[2] With the naked eye the aortic lining shows pale raised lesions, especially in the upper part, and after prolonged feeding of the cholesterol-rich diet, most of the aorta, as well as parts of other arteries, may become affected. With the light microscope the lesions are characterized by the development of multilayered cushions of lipid-filled cells ("foam cells"), a variable amount of necrosis, collagen fibrillogenesis, production of acid mucopolysaccharide and calcification. The present communication describes the fine structure of the endothelial lesions.

Methods

Male rabbits of 2 to 3 kg. body weight were fed a diet containing 1.5 per cent of cholesterol (U.S.P.) intimately mixed with the rabbit food pellets (Master Feeds) by dissolving it in ether which was then evaporated. Rabbits were kept on this diet for periods of 14 days, 1, 2, 3, 5 and 7 months. Each of 3 rabbits, sacrificed after 2, 3 and 5 months on the diet, was given an intravenous injection of 5 ml. of colloidal thorium dioxide (Thorotrast; Testagar and Co.) 24 hours before sacrifice. Thirteen rabbits were given this diet. Three other rabbits received the same amount of cholesterol in their diets, but 5 per cent corn oil was added; the animals were sacrificed after 6 months. Although the fine structure of normal rabbit arterial endothelium has been reported elsewhere,[3] some of its important features are reviewed here, by way of introduction to the observations in atheromatous vessels.

The thoracic aorta and sometimes the femoral artery were fixed in osmium tetroxide, dehydrated, and embedded in methacrylate as described previously.[3] Sections were cut on a Porter-Blum microtome

* Supported by a grant from the Life Insurance Medical Research Fund.
Received for publication, February 17, 1958.

and examined with a Philips EM 100 A electron microscope. Frozen sections and paraffin sections of formalin-fixed material were observed with the light microscope.

Observations

Endothelium of Normal Rabbits

The endothelial cells often protruded into the lumen as a consequence of contraction of the artery (Fig. 1). They lay either directly on the internal elastic lamina or on a delicate network of collagen fibrils. The endothelium formed a continuous cellular layer although, in places, it was extremely thin. The shape of the nuclei was determined by the amount of contraction of the vessel and varied from flattened to spherical configuration. The cytoplasm contained relatively few mitochondria, some membranes of a Golgi apparatus, and numerous vesicular or tubular structures of great variety in size. These were thought to be a part of the endoplasmic reticulum. A few dense bodies, about the size of mitochondria, were present and were shown to incorporate intravenously injected particles of thorium dioxide. A striking feature was the very great number of tiny invaginations of the plasma membrane on all surfaces of the cells (Fig. 2). Both Palade[4] and Moore and Ruska,[5] who observed these structures in capillaries and small arteries, considered that these might represent pinocytic vesicles, and might be of importance in the movement of fluid substances through the cell.

Endothelial Lesions in Rabbits Receiving a 1 Per Cent Cholesterol Diet

Animals sacrificed after 14 or 30 days on the diet had no lesions of the aorta visible to the naked eye or the light microscope. The fine structure of the aorta also showed no consistent change from that of normal endothelium. At the end of the second month on the diet, however, lesions were found, and all animals sacrificed after 3, 5, and 7 months showed well-developed experimental atherosclerosis.

The atherosclerotic plaques were always covered by a continuous unicellular layer. The cells were frequently of pyramidal shape, with the base towards the lumen (Figs. 3 and 5). In places, this cell layer was extremely thin, reduced almost to the width of the two cytoplasmic membranes. The nuclei were oval or circular in outline. The cell membrane was folded at the lateral intercellular boundaries (Fig. 4) and a light gap 100 to 200 Å wide was enclosed between the membranes of the cells in this layer. Each cell appeared closely united and interdigitated with those on either side of it, but its deep margin was separated from the inner cells of the plaque by a large and irregular

extracellular space (Figs. 3, 4 and 5). Adjacent to the cytoplasmic membrane of all surfaces were small vesicles or invaginations (Fig. 6) of the type found in greater numbers in normal arterial endothelium. They measured up to 600 Å across.

The cytoplasmic matrix was somewhat more dense than that of normal endothelium. It contained large cisternas of endoplasmic reticulum, normal mitochondria, and a much hypertrophied Golgi apparatus. Occasionally, relatively large light spaces and dense granular inclusions were observed. The endoplasmic reticulum (Figs. 4, 5 and 6) was represented by numerous and frequently distended cisternas and zones measuring up to 1 μ^2, filled with moderately dense material. The outer surface of its membranes was studded with particles of about 150 Å size, the Palade granules (Fig. 6).

Parts of the Golgi apparatus were usually seen at several points in a section of each cell (Figs. 4 and 6). The structure was much more developed than in normal endothelium, where it was relatively inconspicuous. It consisted of circular profiles 300 to 700 Å in diameter, parallel membranes which appeared to be oblique, and longitudinal sections through tubules or cisternas. The membrane bounding these structures had a smooth surface, in contrast to the endoplasmic reticulum.

Cytoplasmic inclusions were of two types. The commonest, which was also found in normal endothelium, consisted of relatively dense material bounded by a single membrane (Fig. 4). Another type of inclusion, seen only occasionally, was larger and appeared as an empty space, often having no definite limiting membrane.

Twenty-four hours after the intravenous injection of Thorotrast, some of this material could be seen in the first type of inclusion described above. In some inclusions only a few dense particles were present, but in others so many were taken up that it was not possible to see whether they were, in fact, lying in the dense matrix of this inclusion or whether they had formed a different type of vacuole, containing only the thorium particles (Fig. 8).

The interior of the lesions contained cells, relatively large extracellular spaces, and fibrils of collagen. The cells were of two types. Those making up the covering layer were occasionally found close to the surface of the lesions (Fig. 3). This type of cell, which was characterized by prominent cisternas of endoplasmic reticulum and the virtual absence of vacuoles, was much less numerous than the other. The latter appeared to correspond to what conventional microscopists have called "foam cells" (Figs. 5 and 7). The cytoplasm in these contained many light spherical vacuoles, usually having definite limiting

membranes (Fig. 5). Typically, these cells were more or less spherical, measuring 15 to 40 μ in diameter. Sometimes they contained more than one nucleus. The cytoplasm between the light vacuoles contained mitochondria, irregularly oval profiles of endoplasmic reticulum and, occasionally, numerous very dense granules. The endoplasmic reticulum did not show the large cisternas of the surface cells. The very dense granules usually appeared to be associated with the light vacuoles (Fig. 5).

Parts of the margins of these cells did not have contact with other cells, so that extracellular spaces of irregular shape were formed (Figs. 3 and 7). Such spaces contained fibrils assumed to be collagen and much finely granular material. The substance in which fibrils and granules were embedded was of low electron density. The proportion of fibrils and other extracellular material to cells appeared to increase with increasing time on the diet.

Single-layered endothelium was found between the atherosclerotic plaques. With the naked eye, the conventional microscope, and the electron microscope, many portions of this endothelium retained the normal structure, even after 7 months on the diet. Other regions showed definite pathologic alterations in the form of slight increase in prominence of the endoplasmic reticulum and Golgi apparatus and the appearance of irregular dense masses in the cytoplasm. The small invaginations of the surface membrane were present, as were larger pockets projecting into the cytoplasm. These features of the cell membrane were found also in normal endothelium.

Endothelial Lesions of Rabbits Receiving a Diet of 1 Per Cent Cholesterol and 5 Per Cent Corn Oil

Vacuoles (Fig. 9) were seen more frequently in the surface layer of cells than in the corresponding cells of the rabbits on cholesterol diet only. The vacuoles now appeared to contain very dense material. Similarly, in the cells in the interior of the lesion (Fig. 9), many dense bodies and a smaller number of light ones were present. The former were interpreted to be osmiophilic lipid vacuoles and the latter to represent either non-osmiophilic lipid or some other material. In other respects the lesions in these arteries resembled those already described.

Discussion

The chemical nature of substances contained in the various inclusions could not be defined by electron microscopy. The lesions of cholesterol-fed rabbits have been shown to contain lipids by Sudan staining[6] and by chemical analysis.[7] Moreover, minerals,[8] acid muco-

polysaccharides[9] and collagen[6] have also been found in them. It is usually assumed that the lesions develop as a result of the incorporation into endothelial cells of substances from the blood in the lumen, although Leary[10] proposed that they might be formed by the deposition of "foam cells," as such, on the endothelial surface. A high level of blood lipids is associated with the development of the lesions, and the lipid has been shown to be combined with protein.[11]

This background of earlier work on experimental cholesterol atherosclerosis is of limited value in assessing the significance of the observations by electron microscopy. The problem is, of course, to localize, in the lesion, these various substances which have been identified by chemical methods. As far as I am aware, no study of the cytochemistry (except for Sudan staining) of these lesions has yet been published.

Lipoidal materials may appear as dense granules after osmium fixation (corn oil in intestinal cells[12]) or may be dissolved out in the preparation of the sections (sebaceous gland cells[13]), giving the cells a "foamy" or vacuolated appearance. Which of these two forms the lipid assumes in the sections appears to depend largely on the degree of saturation; unsaturated lipids generally become blackened by osmium.[14] Lipoprotein usually has a high density in electron micrographs (plasma membrane, mitochondria, myelin sheath). A paper by Wigglesworth[15] should be consulted for a more detailed account of osmium in fixation of lipids.

In the present study, frozen sections of formalin-fixed tissue were stained with Sudan black B in an attempt to correlate the electron micrographs with the features observed with the light microscope. The staining was diffuse, and a clear distinction could not be made between the surface covering cells and those in the interior of the plaque. Both the cells and the extracellular material were stained. The impression was gained, however, that the vacuolated cells noted in the interior of the plaques in the electron micrographs were probably "foam cells" and that the "empty" areas were spaces from which lipid had been dissolved. It was to test this idea that the rabbits were given the added corn oil, an unsaturated lipid, rather than cholesterol alone, on the assumption that osmiophilic lipid would then appear in the lesions. In these animals, many of the vacuoles were found to be filled with dense material, interpreted to be osmiophilic lipid. It was thus concluded that the vacuolated cells in the interior of the lesions in animals receiving cholesterol without corn oil were, in fact, "foam cells" and that probably the majority of the vacuoles contained lipid in life. The particular lipid mixture in these cells appeared to have been largely dissolved in the preparation of the sections, although the dense granules

associated with the otherwise "empty" vacuoles (Fig. 5) could be a highly osmiophilic residue of the vacuole contents. The view that these granules might represent early mineralization is considered with favor although there is no very good evidence for this.

The striking feature in the covering layer of cells was the scarcity of lipid vacuoles of the type seen in the "foam cells." Instead, the dilated cisternas of the endoplasmic reticulum were filled with a moderately dense material. The nature of this material is a matter for speculation at the present time. Since these cells were diffusely stained by Sudan black B, they probably contained some lipoidal material. As a working hypothesis, it is suggested that the substance staining in this manner corresponds to the dense material observed in the electron micrographs and that this is lipoprotein—more specifically, lipoprotein from the plasma.

It is suggested that the layer of cells covering the lesions is actively engaged in the taking up of lipoprotein from the plasma. The obvious hypertrophy of the endoplasmic reticulum and Golgi apparatus, as compared with the normal, speaks for an increased metabolic activity in these cells. The scarcity of lipid vacuoles in them suggests that, in cholesterol-fed rabbits, this layer of cells is presented with plasma lipids in a particle size too small to be recognized in the electron micrographs; that is, in the form of lipoprotein molecules, rather than chylomicrons. This idea is in accord with the observations obtained by ultracentrifugal investigations of plasma lipids in cholesterol-fed rabbits.[11] Possibly, if the cells had larger lipid particles available to them, these would also be taken in. The lining cells are capable of phagocytosis, as seen by their uptake of intravenously administered colloidal thorium dioxide. The conventional microscopic observations of Duff, McMillan and Lautsch[16] also established the phagocytic property of endothelial cells.

A hypothesis to cover the present findings is suggested. Lipoprotein enters the surface cells from the lumen. Possibly this occurs by way of the small vesicles under the plasma membrane, although these structures are less numerous than in normal endothelial cells. Lipoprotein is taken into the large cisternas of the endoplasmic reticulum. Here the protein component survives the treatment of embedding in methacrylate and appears in relatively dense fashion in electron micrographs. As the covering layer of cells is only poorly united with those below it (although its cells fit sufficiently closely to form a continuous layer), lipid is passed through the outer cytoplasmic membrane and comes to lie in the relatively wide extracellular spaces. From here it is taken up through a process of phagocytosis by the cells ("foam cells") com-

prising the greater part of the lesion. The location of the lipid is represented by "empty" spaces or vacuoles as the protein previously combined with the lipid has now been separated. Whether these phagocytic cells arise from endothelium or from the macrophage system is not known.

Endothelial lesions produced in the aortas of rabbits by feeding a diet containing 1.5 per cent cholesterol for various periods of time were studied in thin sections with the electron microscope.

Summary

Two morphologically distinct types of cells were observed. The cells of one type formed a continuous layer over the surface of the lesions. This layer was apparently rather loosely bound to the underlying lesion. Peculiar features of these cells were the prominent Golgi apparatus and the dilated cisternas of the endoplasmic reticulum filled with moderately dense material. Only very occasionally were vacuoles seen in the cytoplasm. The other cell type, found in the interior of the lesion, was highly vacuolated.

The electron microscopic picture is interpreted as suggesting the incorporation of lipoprotein into the endoplasmic reticulum of the surface cell layers. It is proposed that this layer of cells may play a particularly important role in the metabolism of lipid in the lesion. The other cell type appears to have the less specific role of phagocytosis of lipid from the extracellular spaces into cytoplasmic vacuoles.

The author is indebted to Mr. William Daniels and Mr. Charles Jarvis for technical assistance. A grant from the Atkinson Foundation, Toronto, for the purchase of the electron microscope is gratefully acknowledged.

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LEGENDS FOR FIGURES

Key:

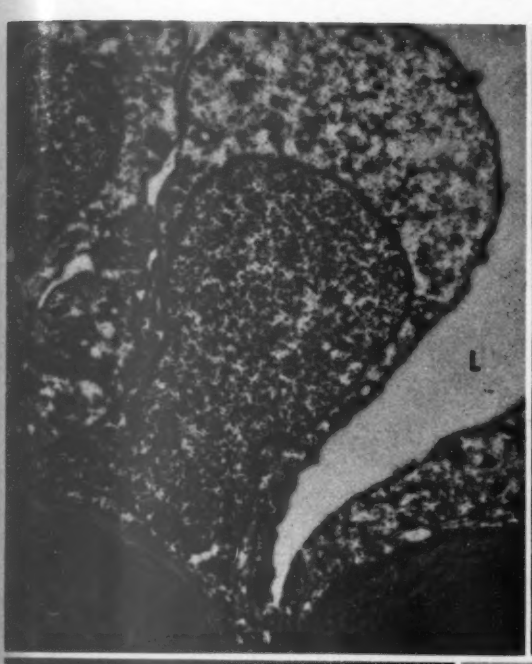
L, lumen of artery
N, nucleus
EL, internal elastic lamina
I, inclusion
V, vesicle or small invagination of cell membrane
GA, Golgi apparatus
ER, endoplasmic reticulum
M, mitochondrion
G, granule
Va, vacuole
ECS, extracellular space
PM, cytoplasmic membrane

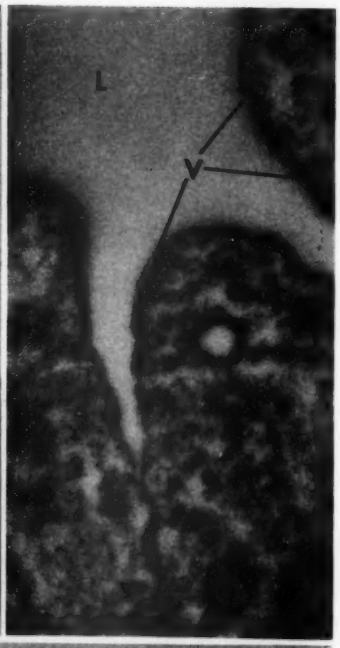
FIG. 1. Normal endothelium of rabbit femoral artery. The cells protrude into the lumen as a result of contraction of the artery. They may rest directly on the internal elastic lamina, as seen here, or on a network of a few collagen fibrils. × 13,000.

FIG. 2. In the normal endothelial cell, the most characteristic feature of the cytoplasm is the presence of numerous tiny vesicles which are invaginations of the cytoplasmic membrane. × 41,000.

FIG. 3. Survey micrograph of a part of an intimal lesion from the aorta of a rabbit on a high cholesterol diet for 7 months. This section shows the characteristic pyramidal surface cell and part of the cytoplasm of two cells deeper in the lesion. One cell (a) is the common type in this location, having many light cytoplasmic vacuoles. The other (b) resembles the surface-type cell in having few vacuoles but a prominent endoplasmic reticulum. A large part of the lesion consists of extracellular material. × 9,000.

FIG. 4. A small area at the surface, showing the junction between two covering cells and a small part of one of the underlying cells (lower right quadrant). The covering cells are closely fitted at their lateral margins but not on their deep surfaces. The cytoplasmic membranes between adjoining cells of the surface layer may be quite convoluted, as seen here. The Golgi apparatus of these cells, consisting of smooth surfaced membranes, is prominent and here is seen cut across at two places. × 23,000.





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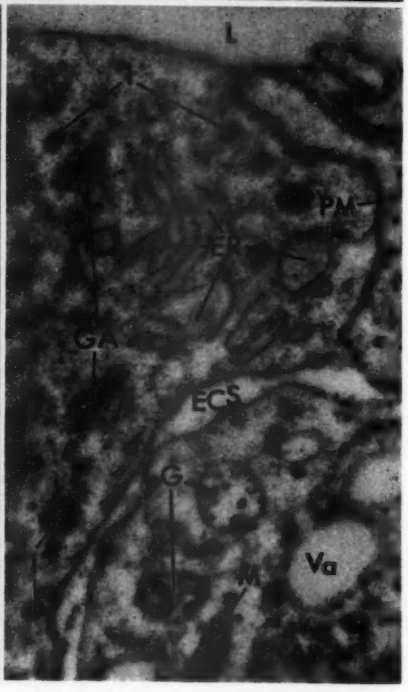

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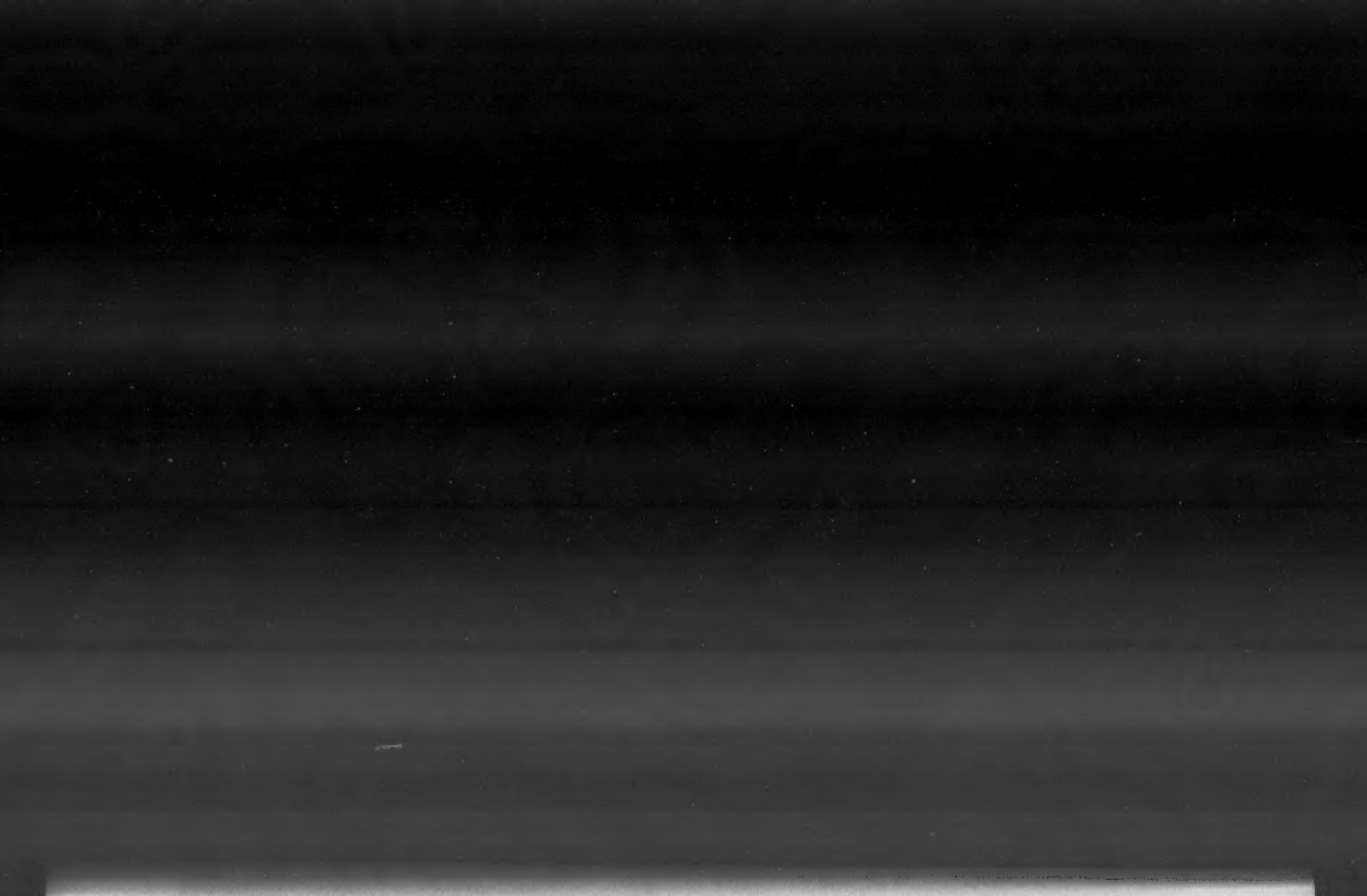
FIGS. 5 and 6. Figure 6 is the area outlined in Figure 5. The layer of covering cells containing prominent Golgi apparatuses and the dilated cisternas of the endoplasmic reticulum are covered on their outer surfaces by many fine granules (Fig. 6). Small invaginations of the cell membrane are seen at the surface and at intercellular boundaries. The cytoplasmic membranes between cells of the covering layer are separated by a relatively uniform gap, which enlarges to a wide and irregular extracellular space under this layer. This space continues into the interior of the lesion. A portion of one of the cells of the type found in the interior of the lesion is seen in the lower left quadrant of Figure 5. It contains many clear vacuoles, some of which are associated with very dense granules. Rabbit on cholesterol-rich diet for 7 months. Fig. 5: × 18,000; Fig. 6: × 39,000.



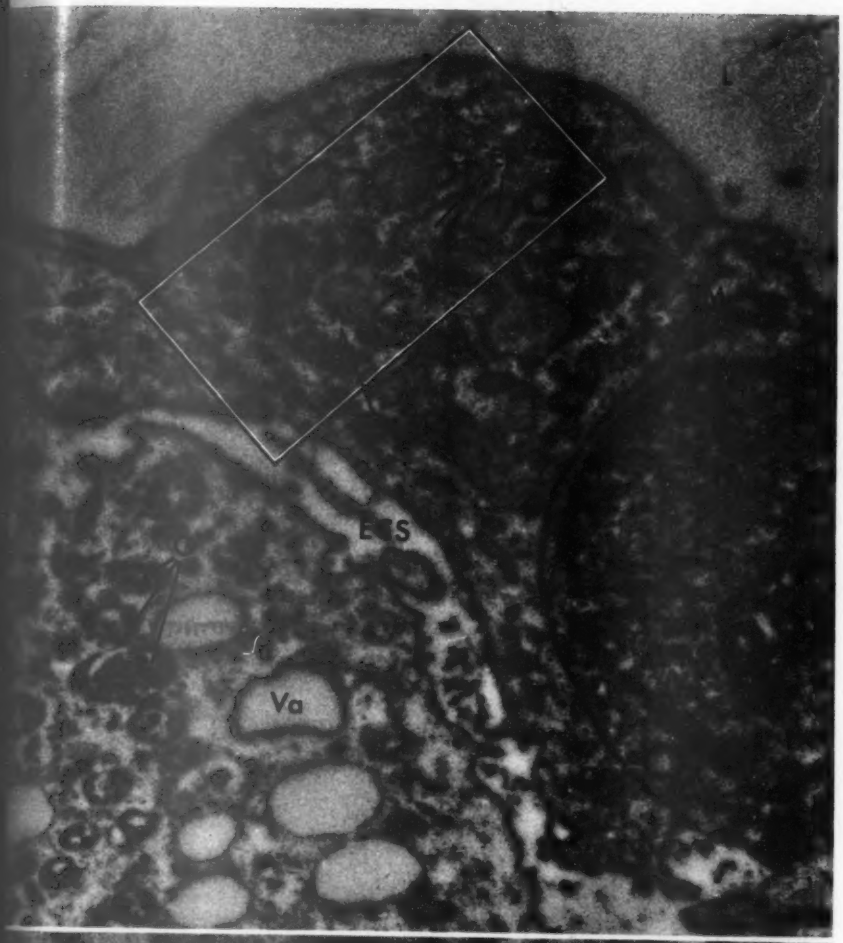


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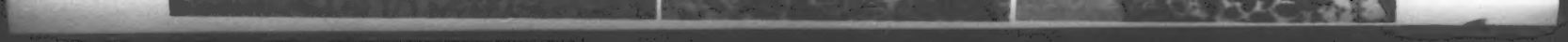
FIG. 7. Rabbit on high cholesterol diet for 5 months. Interior of a large plaque, showing two of the large, highly vacuolated cells interpreted as "foam cells." The extracellular space contains granular material and, in places, fibrils of collagen. × 6,000.

FIG. 8. Surface cell of lesion from a rabbit on high cholesterol diet for 5 months. An intravenous injection of 5 ml. of Thorotrast was given 24 hours before sacrifice. Particles of thorium dioxide are packed into cytoplasmic inclusions. × 22,000.

FIG. 9. Aorta of a rabbit fed 1.5 per cent cholesterol and 5 per cent corn oil for 6 months. In the surface layer of cells are vacuoles containing dense material, interpreted as osmiophilic lipid. Vacuoles of two types are found in other cells of the lesion; i.e., dense vacuoles, considered to be osmiophilic lipid droplets, are scattered among some light vacuoles in the cytoplasm of two cells below the surface layer (lower left and lower right). × 8,000.



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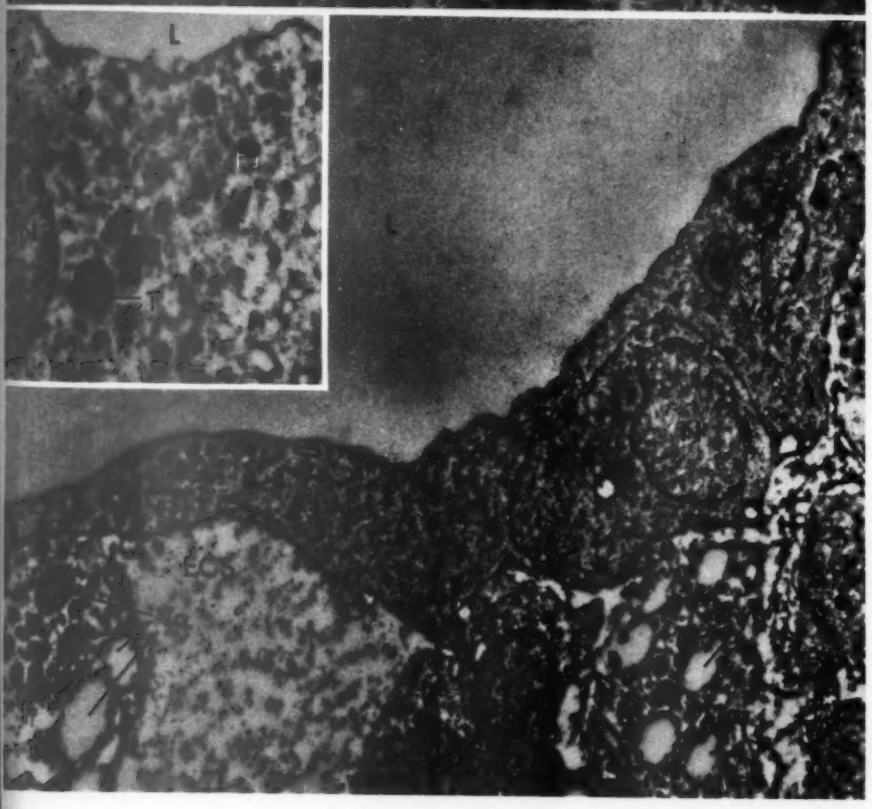

9

THE PROPERDIN SYSTEM IN TRANSPLANTABLE CANCER

(GARDNER LYMPHOSARCOMA 6C3HED)*

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In recording our experiences with heterologous transplantation of human tumors, we[1] postulated that the natural defensive mechanism of animals against implanted neoplasms, and for that matter against foreign tissue of any type, might be connected with the properdin system. This theory was derived from (a) reports by Murphy[2] in 1914 and Toolan[3] in 1951 that the innate resistance of animals to implanted foreign tumors could be penetrated by total body irradiation; (b) the disclosure by Kidd[4] in 1953 that normal guinea pig serum contains a "protein" capable of producing complete regression of lymphosarcoma 6C3HED in C3H mice; and (c) the discovery by Pillemer and coworkers[5] in 1954 of a new euglobulin in serum which they called properdin. This protein, in combination with complement and Mg^{++}, is believed to be responsible for natural immunity in the animal body. It destroys bacteria, neutralizes viruses, causes lysis of erythrocytes, and is severely depleted in animals by total body irradiation. In view of the facts listed, and because of the nonspecificity and wide range of activity of properdin, we[1] theorized (a) that the conditioning of animals for implantation of foreign tumors by irradiation may be due to destruction of properdin, (b) that the tumor inhibitory "protein" in guinea pig serum discovered by Kidd[4] may actually be properdin, and (c) that properdin may be the natural tumor inhibitor in the animal body.

Since properdin was unobtainable, we[6] elected to use zymosan in an effort to investigate the possible role of the properdin system in transplantable cancer. Zymosan is an insoluble carbohydrate complex derived from yeast cell walls which is capable of combining with properdin both *in vitro* and *in vivo*, thus influencing the properdin level of serum and blood.[7] By actual measurement, Pillemer and coworkers[5] had shown that total body irradiation of experimental animals was followed by severe depletion of the properdin level in the blood. Pillemer and Ross[8] demonstrated that intravenous injections of small amounts of zymosan produced an early fall (below normal) and then a rapid rise (above normal) of properdin titer in the blood

* Received for publication, January 11, 1958.

while large doses resulted in continued depression of properdin levels. Using a transplantable adenocarcinoma (HR132) from a human colon and weanling Wistar rats, we[6] were able to show that (a) nontreated animals (with presumably normal blood properdin levels) exhibited poor tumor "takes" (19 out of 160); (b) irradiated animals (with presumably markedly depressed blood properdin levels) showed excellent tumor "takes" (136 out of 160); (c) animals given a single injection of zymosan (with presumably slightly depressed blood properdin levels) revealed fair tumor "takes" (58 out of 160); and (d) animals given multiple injections of zymosan (with presumably moderately depressed blood properdin levels) disclosed good tumor "takes" (90 out of 160). Since the properdin levels were inferred from the work of Pillemer and Ross[8] and not actually measured, we concluded that zymosan was effective in increasing the susceptibility of weanling Wistar rats to the HR132 carcinoma. Whether this conditioning was mediated through the properdin system remained to be proved.

The purpose of this report is to record additional findings in connection with the possible role of the properdin system in transplantable cancer.

Materials and Methods

The tumor-host combination used in all experiments consisted of the Gardner lymphosarcoma 6C3HED and young female C3H mice, each weighing approximately 20 gm. Most of the animals (840) were purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and only a few (36) were procured from Rockland Farms, New City, New York. The donor tumor was 9 days old. Transplantation was accomplished by trocar introduction into the subcutaneous tissues of the right flank. Treatment was begun on the fourth day, at which time the tumors measured approximately 8 mm. in diameter. All fluids were given intravenously by way of tail veins.

In each experiment a group of animals approximating the number in each test group was set aside as a nontreated control, and a similar number was treated with sufficient (previously determined) concentrated guinea pig serum (conc. g.p.s.) to cause complete regression of tumor. Guinea pig serum was obtained from Cappel Laboratories, West Chester, Pennsylvania, and rat serum was procured from Carworth Farms, New City, New York. All serums were purchased in the lyophilized state and were restored with sterile distilled water in a ratio of 1 ml. being equivalent to $3\frac{1}{3}$ ml. of normal serum. Fleischmann's zymosan was purchased from Standard Brands Incorporated, New York, and was prepared for use by the method of Pillemer and co-workers.[9] Properdin was obtained from, and properdin levels were

determined by, Dr. Louis Pillemer. Total body irradiation (220 KVP, 36 r. per minute, 50 cm. TSD, 1.0 Cu HVL) was administered by Mr. Henry Boudreau in the Department of Radiology.

Animals were weighed at the beginning, several times during the course, and at the end of the experiments. All surviving animals were sacrificed 14 days after implantation of tumor. The end point of the experiment, nevertheless, was taken as the eleventh day, since by this time a clear cut effect upon the tumor could be determined. Necropsies were performed on all animals, and the tumor or tumor site, liver, and one kidney from each mouse were examined microscopically. Since, however, it is impossible to differentiate histologically between tumor lymphocytes and inflammatory lymphocytes which are present after any tumor resorbs, complete gross disappearance of the implanted growths, rather than absence of lymphocytes, was considered indicative of complete regression of tumor.

Results and Discussion

The combined results of the experiments are outlined in Table I. Animals receiving no treatment totaled 197; of these, 185 survived 11 days or longer. Of this group, all but 4 showed progressive growth of tumors, the average diameter of the lesions being 26 mm. The increase in average weight of mice from 22 gm. to 29 gm. was due almost entirely to the tumor itself.

Properdin levels in serums from normal nontumor bearing mice, determined on several occasions, varied between 9 and 18 units per ml. Repeated determinations of levels in mice bearing tumors from 4 to 14 days old varied from 2 to 6 units per ml. In one series, determinations in animals with tumors for 4, 6, 8, 11, and 14 days were 9, 6, 6, 4, and 1 units per ml. respectively. The properdin level in serums from the few mice that first showed growth and then spontaneous regression of tumor was 9 units per ml. Thus, repeated determinations in mice with growth of tumors disclosed depressed properdin titers in their serums while on one occasion, when the tumors regressed spontaneously, the properdin titer was normal.

Animals treated with concentrated guinea pig serum (conc. g.p.s.) uniformly revealed a good tumor inhibitory response. Initially, 1 ml., given on the fourth and sixth days after implantation of tumor, was used and resulted in complete regression of tumor in each of the 26 mice that survived 11 days or longer. Subsequently, graded tolerance doses were employed, revealing that 0.2 ml. given 3 times a week for 5 injections (3 × 's/wk. × 5) was enough to give uniformly good results. In 148 mice so treated that survived 11 days or longer, there were only

TABLE I

Treatment, Number and Size of Tumor "Takes," and Weights of Animals

Treatment	No. mice used	No. mice surviving 11+ days	No. of growths 11+ days	Average diameter tumors (mm.)	Average weight of animals (gm.) Initial	11+ days
None	197	185	181	26	22	29
1 ml. conc. g.p.s. 4 and 6 days after implant.	31	26	0	0	23	25
0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5*	154	148	5	12	22	22
0.1 ml. conc. g.p.s. 3 ×'s/wk. × 5	10	10	9	10	21	17
0.08 ml. conc. g.p.s. 3 ×'s/wk. × 5	15	15	15	21	25	33
0.05 ml. conc. g.p.s. 3 ×'s/wk. × 5	55	55	55	23	22	27
25 units properdin daily × 6	23	20	20	22	23	28
25 units properdin + 0.05 ml. conc. g.p.s. 3 ×'s/wk. × 5	19	19	19	19	24	27
50 units properdin + 0.05 ml. conc. g.p.s. 3 ×'s/wk. × 5	15	15	15	24	20	32
50 units properdin + 0.05 ml. conc. g.p.s. + 0.05 ml. of 1:20 dilution of 50% $MgSO_4$ 3 ×'s/wk. × 5	15	15	15	23	23	23
0.05 ml. conc. g.p.s. + 0.05 ml. of 1:20 dilution of 50% $MgSO_4$ 3 ×'s/wk. × 5	15	15	15	24	24	34
0.05 ml. of 1:20 dilution of 50% $MgSO_4$ 3 ×'s/wk. × 5	15	15	15	26	22	38
0.2 ml. conc. rat serum 3 ×'s/wk. × 5	15	15	15	25	24	35
0.08 ml. conc. rat serum 3 ×'s/wk. × 5	15	15	15	24	24	35
0.08 ml. mixture, equal parts of conc. g.p.s. and rat serum 3 ×'s/wk. × 5	15	15	15	24	23	33
600 r. 3 days before implant. + 0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5	24	7	2	7	24	22
500 r. 1 to 3 days before implant.	61	23	23	17	17	16
500 r. 1 to 3 days before implant. + 0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5	61	18	13	8	19	18
300 r. 1 day before implant.	36	29	29	22	21	23
300 r. 1 day before implant. + 0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5	30	26	0	0	17	18
80 mg. zymosan per kg. body wt. 3 ×'s/wk. × 7, starting 1 day before implant.	31	24	24	23	17	26
80 mg. zymosan per kg. body wt. 3 ×'s/wk. × 7, starting 1 day before implant. + 0.2 ml. conc. g.p.s. 3 ×'s/wk. × 5	24	17	3	11	23	24

*Indicating injections 3 times a week for 5 injections; other abbreviations explained in text.

5 growths, and the average diameter of these (12 mm.) was less than one half that of untreated controls (26 mm.). Injections of 0.1 ml. had a distinct inhibitory effect upon the tumor while injections of 0.05 ml. had no effect and injections of 0.08 ml. were just on the border of producing an inhibitory response. These results clearly indicate that normal guinea pig serum possesses a tumor inhibitory principle (TIP) which causes complete regression of Gardner lymphosarcoma 6C3HED in C3H mice. They thus confirm Kidd's contentions.[4]

Properdin levels in serums from tumor bearing mice treated with concentrated guinea pig serum were likewise determined on several occasions. When tumors had disappeared completely, the properdin levels, determined 10 different times, were consistently found to be between 9 and 40 units per ml., except on 2 occasions when they were 6 and 3 units. In one instance when the tumors failed to disappear, the properdin level was 3 units per ml. In one series, normal nontumor bearing mice were given 0.2 ml. of concentrated guinea pig serum 3 times a week for 5 injections as in the tumor bearing animals. Properdin levels in their serums were 24, 18, 9, and 9 units per ml. (the corresponding normal reading was 9 units per ml.). Thus, it may be stated that the properdin levels of serums procured from tumor bearing mice treated with guinea pig serum varied inversely with the growth of the tumor, and that normal mice treated with concentrated guinea pig serum showed an elevation of their serum properdin titers.

In the theory outlined above, it was postulated that the tumor inhibitory principle (TIP) in guinea pig serum might actually be properdin. If this were so, then the administration of properdin should have had a tumor inhibitory effect. The properdin level of the concentrated g.p.s. used in our experiments (determined on 4 separate occasions) was 12 units per ml. The actual amount of properdin in each 0.2 ml. of concentrated serum, which, given 3 times a week for 5 doses, was enough to induce complete regression of tumor in 148 out of 154 animals treated, was 2.4 units. Administration of 25 units of properdin daily (over 10 times that received in g.p.s.) for 6 days, however, had no tumor inhibitory effect, for there were 20 tumor growths in 20 animals that survived 11 or more days, and the average diameter of the tumors was 22 mm. Since a lack of complement, Mg^{++}, or perhaps other substances may have accounted for the failure of properdin to act, 25 units of properdin mixed with 0.05 ml. of concentrated g.p.s. (a dose insufficient by itself to decrease tumor growth) was administered 3 times a week for 5 injections to 19 tumor bearing mice. The results were negative, for the tumors grew in the 19 animals, and the average diameter of the growths was 19 mm. The last experiment was

repeated, increasing the amount of properdin to 50 units, and the results, as noted in Table I, were again negative. Thinking that the combination of properdin and guinea pig serum might have lacked Mg^{++}, other experiments were conducted using (a) properdin, concentrated g.p.s., and magnesium sulfate ($MgSO_4$); (b) concentrated g.p.s. and $MgSO_4$; and (c) $MgSO_4$ alone. Once again, as noted in Table I, the growth of tumors was unimpeded.

Properdin levels (units per ml.) determined in serums from animals treated with properdin, as outlined above, in experiments conducted on two different occasions, were as follows (numbers are listed respectively): (1) normal mice, 18 and 9 units; (2) tumor bearing mice with large 9 day old tumors, 2 and 3 units; (3) mice after the administration of 25 units of properdin daily for 6 days (sacrificed 2 hours after last injection), tumors large, 12 and 9 units; (4) mice receiving 25 units of properdin plus 0.5 ml. concentrated g.p.s. 3 times a week for 5 doses (sacrificed 24 hours after last injection), tumors large, less than 1 and 9 units; and (5) mice receiving 1 ml. of concentrated g.p.s., 5 and 2 days before sacrifice, showing complete regression of tumor, 40 and 18 units. Thus the properdin levels of serums from animals treated with 25 units of properdin in which tumors grew were at low or low normal values, those in nontreated animals in which the tumors grew were low, and those in animals treated with g.p.s. in which the tumors regressed completely were elevated to levels more than twice normal. While the administration of properdin in doses of 25 units had no inhibitory effect upon the growth of lymphosarcoma 6C_3HED in C_3H mice, neither were their serum properdin titers elevated to levels observed in animals treated with g.p.s. The latter animals showed complete disappearance of tumor. It is conceivable that properdin was used in amounts insufficient to produce an inhibitory effect upon the tumor.

Although administration of properdin, as outlined above, had no tumor inhibitory effect, it was deemed advisable to attack the problem indirectly by using rat serum, which possesses a high properdin content. Accordingly, 15 tumor bearing animals were given 0.2 ml. of concentrated rat serum (with a measured properdin titer of 80 units per ml.) 3 times a week for 5 doses. Tumors grew well in each of the 15 animals that survived 11 days or longer, with the average diameter of the growths being 25 mm. Since rat serum is low in complement, another experiment was carried out, using a mixture of 0.08 ml. of equal parts of concentrated g.p.s. and rat serum 3 times a week for 5 doses. The results (Table I) were identical with those obtained using rat serum alone. Thus, rat serum (high in properdin), either alone or

in combination with g.p.s., disclosed no tumor inhibitory effect. Serums from animals in this group were not saved for properdin titers.

Another indirect method, perhaps, of determining whether the tumor inhibitory effect of g.p.s. was mediated through the properdin system or functioned independently of it, was to destroy or deplete properdin in tumor bearing animals and then to treat them with g.p.s. One manner of accomplishing this is exposure of the mice to irradiation. Accordingly, 24 mice were subjected to 600 r. total body irradiation 3 days before implantation of tumor. Four days after the implantation, the mice were treated with 0.2 ml. concentrated g.p.s. 3 times a week for 5 doses. Only 7 mice survived 11 days or longer, and of these, 2 showed tumors, each with a diameter of 7 mm. (Table I). Reducing the preliminary irradiation to 500 r. resulted in a survival of 18 mice out of 61, with 13 showing tumor growth. Each of the tumors measured 8 mm. in diameter. Because 600 r. and 500 r. produced severe irradiation sickness, an additional 30 mice were treated with 300 r. and given g.p.s. as before. Of these, 26 survived 11 days or longer (4 were sacrificed for properdin level determinations), and each of these disclosed complete regression of tumor. Thus, the tumor inhibitory action of g.p.s. was not impeded at all by preliminary treatment with 300 r. and was only moderately affected by exposure to 500 r. and 600 r. Animals in the latter group, however, were sick from the onset of treatment with g.p.s., and too much credence may not, therefore, be placed on the results obtained.

Properdin levels in serums from nontumor bearing animals exposed to 500 r. total body irradiation, taken on days 1, 5, 7, 9, 12, 14, and 15, were 18, 12, 12, 6, 6, 6, and 3 units per ml. respectively. Those from mice exposed to 500 r. and bearing large tumors varied between 3 and 6 units per ml. Similarly treated animals, receiving g.p.s. and showing complete regression of tumor, revealed properdin levels of 12 and 18 units per ml. while those exhibiting some retardation of growth disclosed levels of 9 and 12 units per ml. Animals exposed to 600 r., treated with g.p.s., and showing continued tumor growth, revealed serum properdin levels of 3 and 3 units per ml. On the other hand, in the same experiment, those mice in which complete regression of tumor occurred disclosed levels of 12 and 9 units per ml. Finally, properdin levels in serums procured from (a) nontumor bearing animals 1, 5, 7, 9, 12, and 15 days after exposure to 300 r. total body irradiation were 9, 6, 3, 3, 3, and 3 units per ml. respectively; (b) animals bearing large tumors 1, 5, 7, 9, 12, and 15 days after exposure to 300 r. total body irradiation were 9, 4, 4, 4, 4, and 3 units per ml. respectively; and (c) animals 7, 9, 12, and 15 days after exposure to 300 r. but also

given concentrated g.p.s. with complete regresson of tumor, were 9, 12, 12, and 12 units per ml.

These findings show that the tumor inhibitory principle (TIP) in g.p.s. exerts its influence on the growth of lymphosarcoma 6C3HED despite depression of the properdin system by total body irradiation. They do not, however, indicate that the action of TIP is not mediated through the properdin system. Although the mice were exposed to total body irradiation, the properdin system was still functioning, as indicated by properdin titers not lower than 3 units per ml. in serums from irradiated nontumor bearing animals, and properdin titers as high as 18 units per ml. in serums from irradiated tumor bearing animals treated with g.p.s. and showing retardation or complete regression of tumor. In other words, because of the latter observation, and because properdin titers in serums from irradiated animals in which the tumors grew were low, it is theoretically possible (but certainly not proved) that TIP may exert its influence by way of the animal's own properdin system.

Another method of interfering with the animal's production of properdin before institution of therapy with g.p.s. could be accomplished by giving zymosan in doses large enough to depress serum properdin levels. Accordingly, 31 tumor bearing mice received 80 mg. of zymosan per kg. of body weight 3 times a week for 7 doses, beginning 1 day before the implantation of the experimental tumor. Twenty-four animals survived 11 days or longer, and each of these showed tumors that measured 23 mm. in diameter. At the same time, 24 tumor bearing mice were similarly treated with zymosan but in addition received 0.2 ml. concentrated g.p.s. 3 times a week for 5 doses. In this group, 17 animals survived 11 days or longer, and of these, only 3 showed tumors, each measuring 11 mm. in diameter. Thus, zymosan did not interfere with the tumor inhibitory action of g.p.s.

Properdin levels in serums obtained from nontumor bearing animals treated with 80 mg. of zymosan per kg. of body weight 2 times a week for 4 doses were 6, 12, 6, 3, 18, 6, and 12 units per ml. The low level in each instance was preceded by an injection of zymosan 24 hours earlier. Because the properdin levels were not consistently depressed, zymosan, in subsequent experiments, was given more frequently. Thus, the properdin level in serums from 5 tumor bearing animals treated with 80 mg. of zymosan per kg. 3 times a week and exhibiting large tumors was 3 units per ml. in each instance, while the value in similarly treated animals receiving, in addition, 0.2 ml. concentrated g.p.s. and showing complete regression of tumors ranged from less than 1 to 3 units per ml. Each injection of g.p.s. was administered 4 hours after an

injection of zymosan, thus insuring a low properdin level at the time the g.p.s. was given. Despite this, the tumor inhibitory activity of g.p.s. was unimpeded. Since, however, the properdin system was not completely neutralized, as indicated by measurable properdin titers in the serums, the same explanation may hold as was put forth for animals exposed to irradiation. The one difference in this experiment, as compared with others, is that the properdin titers of serums from animals showing complete regression of tumors were low and not normal or elevated. A possible explanation for this may be that excessive amounts of zymosan in the animal body may have combined with properdin, thus reducing its level in the circulating blood.

Summary

The possible influence of the properdin system in transplantable cancer was investigated by noting the tumor inhibitory effects of combinations of guinea pig serum, properdin, $MgSO_4$, rat serum, irradiation, and zymosan on the growth of lymphosarcoma 6C3HED in C3H mice.

The results indicate the following: (1) Tumor growth in nontreated mice was excellent. (2) The tumor inhibitory response of adequate doses of guinea pig serum was also excellent. (3) Properdin (probably used in inadequate amounts) exhibited no tumor inhibitory response. (4) Properdin combined with smaller amounts of guinea pig serum disclosed no adverse effect on the growth of the transplanted tumor. (5) The addition of $MgSO_4$ to a combination of properdin and guinea pig serum resulted in no inhibition of tumor growth. (6) Rat serum, which is high in properdin, combined with noninhibitory amounts of guinea pig serum, failed to prevent tumor growth. (7) The depression of properdin titers in the circulating blood, induced by total body irradiation or the administration of zymosan, failed to nullify the tumor inhibitory action of guinea pig serum. (8) Normal nontumor bearing mice treated with guinea pig serum showed an elevation of properdin titers in their serums.

Determinations of properdin titers in serums from animals in the various groups indicate, in general, that properdin levels were low when the tumors grew and high when they regressed. The titers bore no relation to the type of treatment administered.

From the results obtained, it may be concluded that the tumor inhibitory principle (TIP) in guinea pig serum is probably not properdin, and that properdin titers of serums from C3H mice vary inversely with the growth of Gardner lymphosarcoma 6C3HED. Whether TIP exerts its influence by way of the animal's own properdin system or is

independent of it, and whether the fluctuations in properdin levels simply represent a reflection of tumor growth rather than its determinant constitute problems which remain to be solved.

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CHEMICAL MEDIATORS IN RELATION TO CYTOLOGIC CONSTITUENTS IN INFLAMMATION*

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In the last 20 years, a number of chemical factors have been identified in inflammatory exudates, which offer a reasonable explanation for the various biologic manifestations of inflammation.[1,2] There is essentially no information concerning the origin of these chemical mediators. It would be of interest, therefore, to determine whether these factors or their precursors can be recovered in the various constituents of injured cells. The generally accepted method of differential centrifugation has been selected to study this problem.[3,4] The observations to be reported indicate the close association of the biologic factors concerned in inflammation with one or several cytologic constituents of injured cells.

METHOD

An acute inflammatory reaction was induced in the right pleural cavity of dogs by the introduction, under pentobarbital anesthesia, of either 1.5 ml. of turpentine or 0.5 ml. of 5 per cent croton oil in olive oil. Both the exudate and the inflamed tissues were removed, usually within 24 hours, and subjected to differential centrifugation.

The exudate was first centrifuged at 2,000 r.p.m. for 10 minutes, and the cellular sediment was suspended in 0.25 M sucrose to obtain the "nuclear fraction."[3] The resulting supernatant was then centrifuged in an angle centrifuge, usually at 21,600 $\times$G, for 45 minutes to one hour. The sediment contained the "mitochondrial-microsomal fraction."[3] The latter designation was applied to indicate that no effort was made to separate mitochondria from submicroscopic particles. The supernatant of this centrifugation was designated the "S_1 fraction." These various fractions were then injected intracutaneously into a rabbit. Thereafter, 6 ml. of 1 per cent trypan blue in saline solution was administered intravenously. One hour later the skin sites were excised and fixed in 10 per cent formaldehyde for subsequent microscopic studies.

The inflamed tissue consisted of the inflamed pleura, occasionally

* Aided by grant E-1346, U.S. Public Health Service and by a grant from the Society of Sigma Xi. Presented before the American Society for Experimental Pathology, Chicago, April 16, 1957. This communication is study No. 52 of a series entitled "Studies on Inflammation."

Received for publication, January 24, 1958.

the adherent inflamed pericardial sac, and usually a superficial portion of the injured lung. These tissues, carefully removed under sterile precautions, were teased into small fragments, and suspended in 10 ml. of 0.25 M sucrose previously kept in the cold. The mixture was whirled for several minutes in a Lourdes multimixer and then transferred to a homogenizer (type 4288B, size "C," A. H. Thomas, Philadelphia). The opaque homogenate was decanted into a beaker. The remaining cellular sediment in the homogenizer was vigorously washed and compressed with 10 ml. of the sucrose solution and added to the first homogenate. This was centrifuged for 10 minutes at 2,000 r.p.m. to obtain the "nuclear fraction."[3] The supernatant was centrifuged in a Servall angle centrifuge for 20 minutes at either 16,900 × G or 21,600 ×G. The sediment represented the "mitochondrial fraction."[3] This was suspended in several milliliters of 0.25 M sucrose. The supernatant was centrifuged for about one hour at either 16,900 ×G or 21,600 ×G to obtain the submicroscopic particles or "microsomal fraction."[3] This was likewise suspended in several milliliters of 0.25 M sucrose. The resulting supernatant fraction was termed the "S_2 fraction."[3] All fractions were stored in the refrigerator when not in use. Experiments were also performed at different stages of the pleural inflammation concomitantly with the rise in hydrogen-ion concentration of the exudate.

Results

Factors Concerned with the Mechanism of Increased Capillary Permeability in Inflammation

There are at least two factors in exudative material which appear to participate in the mechanism of increased capillary permeability in inflammation. These are leukotaxine and exudin.[1,2] There is a possibility that there are other factors also, such as those recently described by Miles and Wilhelm[5] and Spector.[6]

The Presence of Exudin in the Centrifuged Fractions of Exudates. Exudin is recovered in the later stages of an acute inflammation when the exudate is acid in character. Its contribution to increased capillary permeability may be repressed by ACTH but not by cortisone.[7,8] In contrast to leukotaxine it fails to induce an appreciable degree of local leukocytic migration within a test period of approximately one hour.

An intradermal injection with 0.5 ml. of each fraction described in the foregoing section was introduced into the abdominal skin of rabbits. The sediments were suspended in 0.25 M sucrose. Following the introduction of trypan blue (6 ml.) into the marginal vein of the ear, a response similar to that noted with exudin[7] was usually observed in the sites injected with the "S_1" and "nuclear" fractions of acid exudate.

The dye accumulated rapidly in cutaneous areas treated with these fractions, whereas no such consistent effect was observed with the "mitochondrial-microsomal" fraction. The results of the experiments are assembled in Table I. A typical experiment is illustrated in Figure

TABLE I

*Exudin * in Fractions Obtained by Differential Centrifugation of Acid Exudates*

Rabbit no.	pH of exudate	Mitochondrial-microsomal fraction	Nuclear fraction	S_1 fraction
29-75	6.8	o	+	+
29-77	6.9			+
29-60	6.8		+	+
29-84	6.75	+	+	+
29-85	6.2		+	+
29-65	6.8		trace	+
29-82	6.8	trace	+	trace
29-80	6.8	o		+

* The presence of exudin is demonstrated by local increase of capillary permeability, and its repression by ACTH but not by cortisone.

1. There is discrete accumulation of dye in areas treated with either the "S_1" or the "nuclear" fractions of an acid exudate (Fig. 1, areas 1 and 4). The addition of ACTH (5 units) to either of these fractions inhibited somewhat the local passage of the dye from the circulation (Fig. 1, areas 2 and 5). On the other hand the addition of 5 mg. of cortisone acetate added to each of the fractions failed to suppress the local accumulation of the dye (Fig. 1, areas 3 and 6). As stated above and in earlier reports,[7,8] the suppression of increased capillary permeability by ACTH and the inability of cortisone to do so are the distinguishing features of exudin. Stained sections of the treated skin areas were examined microscopically. As a rule, the fractions were found to induce no appreciable leukocytic migration during the one hour period of the experiment (Fig. 2).

Exudin appeared to be absent in the centrifuged fractions of the tissue homogenates when the pH of the exudate was at an acid level. However, it was demonstrable in some of the tissue nuclear fractions when the exudate was at an alkaline pH. The "nuclear" fraction, and to some extent the "mitochondrial-microsomal" fraction of an acid exudate may display a typical necrosin reaction. This phase, however, requires further study. It was shown for instance that similar fractions derived from an alkaline or even a neutral exudate failed to display a typical necrosin reaction.

The Association of Leukotaxine with Some of the Cytologic Constituents of Injured Cells. Leukotaxine has been shown by the writer

to increase capillary permeability and to induce rapid migration of polymorphonuclear leukocytes.[1,2,9-11] Subsequently, leukotaxine was demonstrated to be not only chemotactic for granulocytes *in vitro*, but also for mononuclear phagocytes.[2,12] Chemotaxis for the latter cells merely induced a slower response than in the case of the neutrophils. Finally, the biologic effects of leukotaxine were shown to be suppressed by cortisone but not by ACTH.[2,7,8,13,14]

The homogenate of injured cells from the inflamed pleural cavity of dogs was separated into various fractions by differential centrifugation as described previously. The hydrogen-ion concentration of the exudate was measured prior to removal of the inflamed tissue. The "mitochondrial" fraction suspended in 0.25 M sucrose, when injected intracutaneously in amounts of 0.5 ml. into the rabbits, produced both an increase in capillary permeability as indicated by the local accumulation of trypan blue, and a migration of leukocytes as well. These effects were repressed by the preliminary addition of 10 mg. of cortisone acetate to the suspension. On the other hand, the addition of 1.6 mg. of ACTH (Armour) had no detectable influence upon the effectiveness of the "mitochondrial" fraction. The leukotaxine-like effects manifested by the "mitochondrial" fraction occurred only if the exudate was neutral or at an alkaline pH. If the pH was acid, the fraction failed to yield such consistent results (Table II). The chemotactic

TABLE II

*Leukotaxine * in Fractions of Inflamed Tissue Homogenates Obtained by Differential Centrifugation*

Rabbit no.	pH of exudate in inflamed tissue	Mitochondrial fraction	Microsomal fraction	Nuclear fraction	S_2 fraction
44-47	7.9	+	+	+	
44-51	7.45	+	+	o	o*
44-58	7.45	+	+	o	o†
44-80	7.0	+	o	o	+
44-67	6.95	+	o	o	+
44-71	6.85	o	o	o	+‡
44-46	6.75	o	o	+	
44-69	6.6	+	+	o	o

* The biologic criteria for the demonstration of leukotaxine consisted not only in demonstrating local increased capillary permeability and chemotactic activity, but also repression of both of these manifestations by cortisone and not by ACTH.

† These S_2 fractions contained a factor which increased capillary permeability, but was repressed by neither cortisone nor ACTH.

‡ Although leukocytes were found in the cortisone-treated area, these were damaged and shrunken, indicating some repression of activity.

effect of the "mitochondrial" fraction with the exudate at a pH of 7.45 is illustrated in Figure 3. The repression of leukocytic migration when 10 mg. of cortisone acetate was added to the fraction is demonstrated in Figure 4. It was concluded from this series of experiments that leukotaxine appeared to be associated with the "mitochondrial" fraction of inflamed tissue, provided the pH of the exudate was either neutral or at an alkaline level. Differential centrifugation of alkaline exudates also yielded similar leukotaxine effects in association with the "mitochondrial-microsomal" fractions.

When the remaining differential fractions were tested in rabbit skin, the leukotaxine-like effects were not altogether consistent. In most instances, the "microsomal" fraction suspended in 0.25 M sucrose yielded results somewhat similar to those induced by the "mitochondrial" fraction. This was the case in 3 of 4 experiments, as indicated in Table II. Here, also, when the pH of the exudate was below 7.0, the results were unpredictable. Any of the 3 fractions might have no effect or might induce a leukotaxine-like effect in random fashion (Table II).

Finally, it was noted that the intracutaneous injection of 0.5 ml. of the "S_2" fraction derived from the homogenate of inflamed tissue at an alkaline pH, might cause an increased capillary permeability, as measured by the local seepage of trypan blue. The effect of this permeability factor was suppressed by neither cortisone nor ACTH. Chemotaxis, whenever it occurred, was likewise uninhibited by these hormones. The nature of this permeability factor in inflamed tissue (Table II) requires further study. This is now in progress.

Factors Concerned with the Mechanism of Leukocytosis

Exudates contain two leukocytosis-promoting factors which provide satisfactory basis for the mechanism of leukocytosis accompanying many types of inflammation.[1,2,15,19] The various fractions obtained by differential centrifugation of homogenates of inflamed tissue were tested for their ability to promote leukocytosis. The only one capable of increasing the number of circulating leukocytes was the final supernatant or most soluble fraction, "S_2." This fraction, injected into dogs by intracardiac puncture in amounts ranging from 2.5 to 10 ml., induced a prompt rise in the blood leukocyte level. The pH of the exudate *in situ* fluctuated between 6.6 and 7.45, but this fact seemed to be inconsequential so far as the induction of leukocytosis in the blood stream was concerned. It should be recalled, in this connection, that earlier studies demonstrated the presence of a thermolabile leukocytosis-promoting factor (LPF) in alkaline exudates,[2,15-17] whereas acid exudates were found to contain a thermostable leukocytosis-promoting

factor.[2,18,19] It is quite possible that the "S_2" fractions contained both of these factors, depending on the pH of the exudate of the inflamed tissue. The data bearing on these observations appear in Table III. As a rule, hourly white blood cell counts were performed for a period of 5 to 6 hours. In two experiments (dogs #4-45 and #4-47, Table III), the leukocytic levels were measured at about 24 hours. In both dogs leukocytosis was present. Moreover, in one experiment (dog #4-47) boiling the "S_2" fraction failed to eliminate its leukocytic-promoting activity (Table III). It is therefore apparent that in this particular case, the "S_2" fraction contained the thermostable factor.[2,18,19] Furthermore, it has been shown in previous studies that prior to its dissociation, the thermostable leukocytosis factor is closely linked with the leukopenic factor and with the fever factor, pyrexin, in acid exudates.[18-20] It is interesting to note that in the experiment under discussion (dog #4-47, Table III) the boiled "S_2" fraction, when injected into the animal, at first induced a leukopenia accompanied by a rise in temperature (3.2° F.). This suggests that the fraction contained the thermostable leukocytosis factor and also the leukopenic factor, as well as pyrexin. As indicated in Table III, the average rise in the number of circulating leukocytes in the series studied with the "S_2" fraction was 66.8 per cent.

TABLE III

Leukocytosis-promoting Factors in S_2 Fractions from Inflamed Tissue Homogenates

Dog no.	Amount of fraction injected (ml.)	pH of exudate in inflamed tissue	Basal white blood cell count (per mm.³)	Highest white blood cell count per mm.³ within 5–6 hours	Increase in leukocytes %
4-43	6	7.45	11,525	16,650	44.5
4-29	3	7.45	11,575	13,350	15.3
4-49	10	7.0	17,650	32,050	81.6
4-49	2.5*	7.0	7,350	8,400	14.3
4-45	6	6.95	14,025	27,050 (in 24 hrs.)	92.9
4-47	10	6.85	10,800	17,750	64.4
4-47	5*	6.85	5,900†	11,500 (in almost 26 hrs.)	94.9
3-97	10	6.6	10,925	24,700	126.1
Average			11,219	18,931	66.8%

* S_2 fraction boiled and cooled prior to intravascular injection.

† Boiled S_2 fraction during first 2 hours induced a sharp leukopenia of 72%; the level then rose as shown. During the leukopenic phase, the temperature rose 3.2° F.

The other fractions obtained by differential centrifugation of in-

flamed tissue homogenates failed to exhibit leukocytosis-promoting activity. Dogs received intravascular injections of each of the various fractions suspended in 0.25 M sucrose in amounts ranging from 2.5 to 8.0 ml. This was followed by hourly determinations of the white blood cell levels. The data bearing on these observations are assembled in

TABLE IV

Absence of the Leukocytosis-promoting Factors in Some Fractions of Inflamed Tissue Homogenates

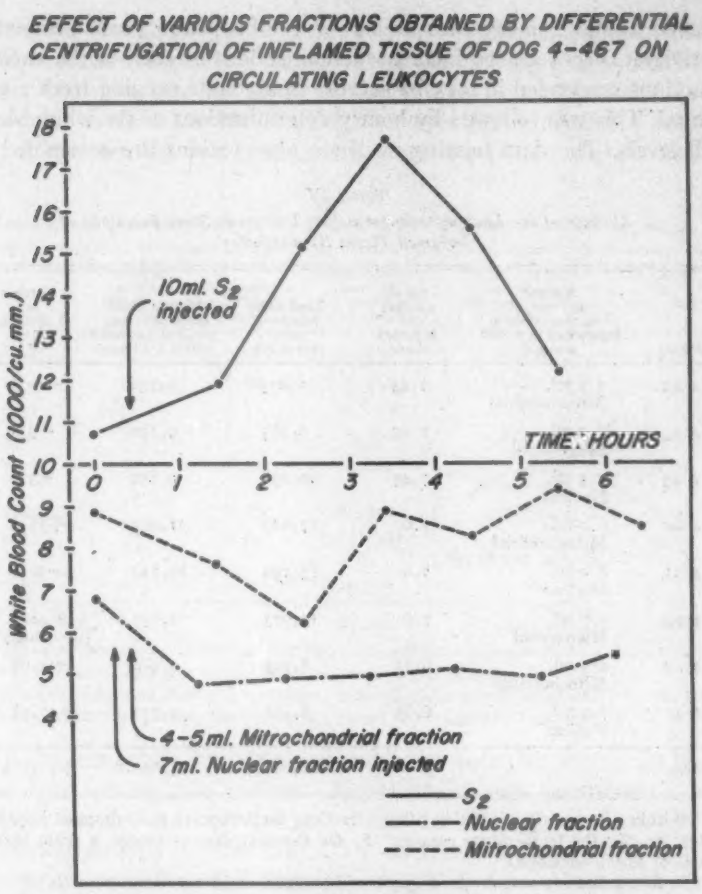
Dog no.	Amount and type of fraction injected (suspended in 0.25M sucrose)	pH of exudate in inflamed tissue	Basal white blood cell count (per mm.^{3})	Highest white blood cell count attained (per mm.^{3} within 1–3 hours)	Changes in leukocytic level (%)
4-43	2.5 ml. Mitochondrial	7.45	10,875	9,750	−10.3
4-43	3.0 ml. Microsomal	7.45	9,875	9,350	−5.3
4-43	5.5 ml. Nuclear	7.45	10,250	9,850	−3.9
4-49	6.0 ml. Mitochondrial	7.0	17,825	21,000	+17.8
4-51	8.0 ml. Nuclear	7.0	13,100	19,750	+50.8
4-49	5.0 ml. Microsomal	7.0	19,075	25,650	+34.5 (in 23 hrs.)*
4-47	4–5 ml. Mitochondrial	6.85	8,925	9,450	+5.9†
4-47	7.0 ml. Nuclear	6.85	6,850	4,750	−30.7‡
Average			12,096.9	13,693.8	+7.35%

* Within 2 hours after injection of this fraction, the leukocyte level dropped approximately 75.4%; the temperature rose 2.2° F. On the next day, as shown, a slight leukocytosis of 34.5% developed.

† In first 2 hours, count dropped 30.5% of base level and temperature rose 1.4° F.

‡ The drop in the leukocytic level was steady within a period of 6 hours, amounting to 24.8% 4 hours after administration of the fraction. The temperature rose 1.6° F.

Table IV. The findings indicated that the leukocytosis-promoting factors appeared to be specifically associated with the supernatant "S_2" fraction alone. A graphic illustration of an experiment appears in Text-figure 1. Since the "S_2" fraction was suspended in 0.25 M sucrose, the effect of this vehicle, administered intravenously, was determined in 2 dogs. No increase in the number of circulating leukocytes of any significance was noted following this maneuver. The increase in white blood cells over a period of 4 to almost 6 hours did not exceed 14.2 per cent and 15.7 per cent respectively. Both of these figures are within the range of normal variation.[15,16]



Text-figure 1. The presence of leukocytosis-promoting factor in "S_2" fraction of inflamed tissue, and its absence in mitochondrial and nuclear fractions.

Factors Concerned with the Mechanism of Fever in Inflammation

In 1944 the writer demonstrated the presence of a pyrogenic substance in inflammatory exudates for the first time.[2,21,22] This substance, termed pyrexin, has been crystallized and is thermostable.[21-23] It has been shown to be recovered primarily from acid exudates. Bennett and Beeson[24] and Atkins and Wood[25] subsequently described an additional thermolabile pyrogen. This was believed to be liberated from neutrophils and might supplement the action of pyrexin. Thus, the presence of two substances would serve to explain the pathogenesis of fever throughout the duration of an inflammatory reaction, from the in-

cipient alkaline stage when neutrophils predominate to the eventual acid stage.[2]

A study was undertaken to determine whether any of the fractions obtained by differential centrifugation exhibited pyrogenic activity. Each of the fractions was introduced into rabbits intravenously, and temperature was measured rectally, usually every 15 minutes for the first hour. Thereafter, measurements were recorded every hour for about 6 hours. The fractions tested were the exudate itself, the "nuclear" fraction, the "S_2" and the "mitochondrial-microsomal" fractions. A summary of the effects of the fractions from exudates appears in Table V. In a study of 23 rabbits, acid exudates were found to contain a pyrogenic factor primarily associated with the "S_2" and the "mitochondrial-microsomal" fractions. In 28 rabbits with neutral or alkaline exudates, the pyrogenic activity was found predominantly in association with the "S_2" fraction (Table V).

TABLE V

Summary of Pyrogenic Activity of Various Fractions Obtained by Differential Centrifugation of Inflammatory Exudates

No. of rabbits	Reaction of exudate: pH range	Average of maximum increase in temperature (°F.)			
		Exudate	Nuclear fraction	S_2 fraction	Mitochondrial-microsomal fraction
23	6.25 – 6.9	2.35	1.5	2.65	2.8
28	7.0 – 7.8	2.0	1.9	2.65	1.7

The pyrogenic activities of fractions of exudate and of the homogenates of inflamed tissue at alkaline or acid pH have been compared. Various investigators have pointed out that there is considerable difficulty in obtaining sharp separation of fractions by the method of differential centrifugation.[4,26] Contamination of fractions with one another is a frequent occurrence.[4] The heterogeneity of some fractions may well explain the appreciable variations which were encountered in this study.

The experiments were conducted as in the foregoing series. Approximately 0.5 ml. of whole exudate or of various fractions derived from either the exudate or the homogenates of inflamed tissue were suspended in 0.25 M sucrose and injected into the ear vein of rabbits. Rectal temperatures were recorded for a period of about 6 hours. The results of a series of such experiments when the exudate was alkaline (pH 7.7) are assembled in Table VI. The exudate was found to be only mildly pyrogenic, yielding a maximum temperature increase of 1.2° F. The "nuclear" fraction of the exudate was found to be about as pyrogenic as the exudate itself, whereas the "nuclear" fraction of

the tissue homogenate was found to be wholly inactive in 2 of 3 tested rabbits. Pyrogenic activity was found in the "S_2" fraction of exudate; an average maximum increase in temperature of 2.15° F. was induced. There was no such consistency of results with the "S_2" fraction of the inflamed tissue homogenate. The "mitochondrial" fractions from both exudate and homogenate were markedly active, resulting in an average maximum increase in temperature of 2.1° F. and 2.3° F. respectively. The "microsomal" fractions provoked only mild fever, the average temperature rise being 1.2° F. with the "microsomal" fraction of exudate and 1.0° F. with that of the homogenate. These variations are believed to indicate a degree of heterogeneity in the fractions themselves (Table VI).

TABLE VI

Comparison of Pyrogenic Activity of Various Fractions of Inflamed Tissue Homogenates with Those of Alkaline Exudate (pH 7.7)

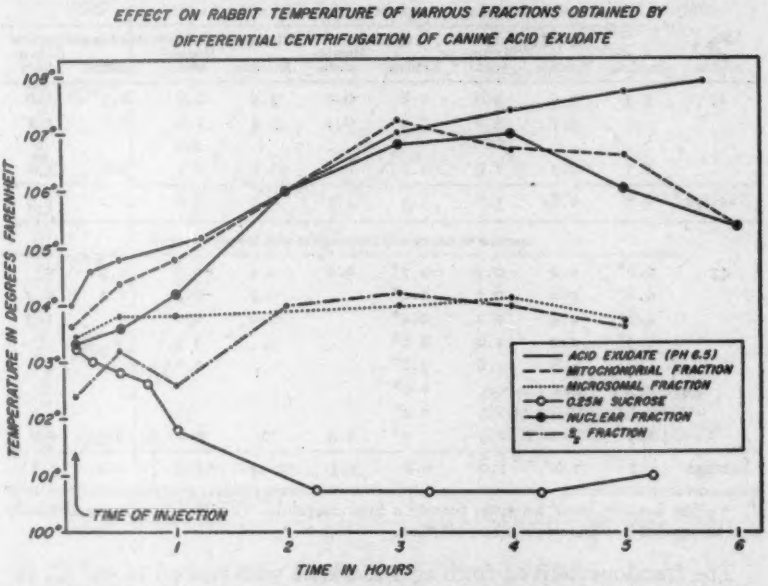
No. of rabbits	Maximum increase in temperature (°F.) within 2 to 6 hours								
		Nuclear fraction		S_2 fraction		Mitochondrial fraction		Microsomal fraction	
	Exudate	Exudate	Homogenate	Exudate	Homogenate	Exudate	Homogenate	Exudate	Homogenate
22	1.2	1.4	1.0	1.7	0	2.4	2.2	−0.1	1.9
		−0.2	−0.5	2.6	1	1.8	2.4	1.2	0.5
		1.8	−0.2					1.5	0.6
								2.3	
Average	1.2	1.0	0.1	2.15	0.5	2.1	2.3	1.2	1.0
	Maximum increase in temperature with heated fractions								
8				0.5*	0.3	1.2	1.6		
				1.4*	0.6	1.6	1.4		
Average				0.95	0.45	1.4	1.5		

* This fraction formed a coagulum upon heating. Two ml. of physiologic saline solution was added, and the coagulum was broken up mechanically.

The heat stability of the two most active fractions ("S_2" and "mitochondrial") was tested by heating each to 90° C. for 30 minutes. In the case of the "S_2" of exudate, the pyrogenic activity was found to be markedly reduced; that of the "mitochondrial" fraction was only slightly affected. An average reduction of 2.15° F. to 0.95° F. occurred after heating the "S_2" fractions, while heating both "mitochondrial" fractions was followed by an average decrease of only 0.7° F. and 0.8° F. (Table VI). These observations suggest the presence of a thermolabile, as well as a thermostable pyrogenic factor. The latter, especially that in the "mitochondrial" fraction, may very well be pyrexin, whereas the former may yet prove to be similar in nature to the factor recently reported by Bennett and Beeson.[2,21,22,24] The

thermolabile factor appeared to be particularly conspicuous in the "S_2" fraction of exudate (Table VI).

A series of experiments was also undertaken to compare the pyrogenic activity elicited by acid exudates with that evoked by the corresponding centrifuged fractions of exudates and inflamed tissue. The inflammatory exudate has an acid pH in the later stages of a developing acute inflammation.[2,27,28] As in the foregoing experiments, utilizing fractions of alkaline exudates, 0.5 ml. of acid exudate at pH 6.7 was injected intravenously into rabbits and the rectal temperature recorded at intervals for several hours. The "nuclear," "S_2," "mitochondrial" and "microsomal" fractions, obtained from both exudative material and homogenates of inflamed tissue by a method similar to that de-



Text-figure 2. Effect of fractions derived from a canine acid exudate on rabbit temperature. Note the absence of any pyrogenic effect by 0.25 M sucrose, the suspension vehicle.

scribed for liver homogenates by Schneider, Hogeboom and Kuff,[3,4] were also injected intravenously into rabbits. The various fractions were suspended in 0.25 M sucrose which is nonpyrogenic *per se* (Text-fig. 2). Seventy-two rabbits were employed in this series of experiments. The data of all observations are assembled in Table VII. The levels of maximum temperature rise indicated that acid exudates were markedly pyrogenic, the average increase being 2.8° F. The "nuclear"

fractions of both acid exudates and tissue homogenates were markedly pyrogenic, yielding an average maximum temperature rise of 2.8° F. and 3.6° F. respectively. The "mitochondrial" fractions were also quite active, inducing an average rise of 2.3° F. and 2.6° F. respectively. In general, all the cytologic fractions exhibited a degree of pyrogenic activity, although the average rise in temperature provoked by the "S_2" fraction was not as great as in the case of the other fractions studied (Table VII). The course of these experiments is illustrated in Text-figure 2.

TABLE VII

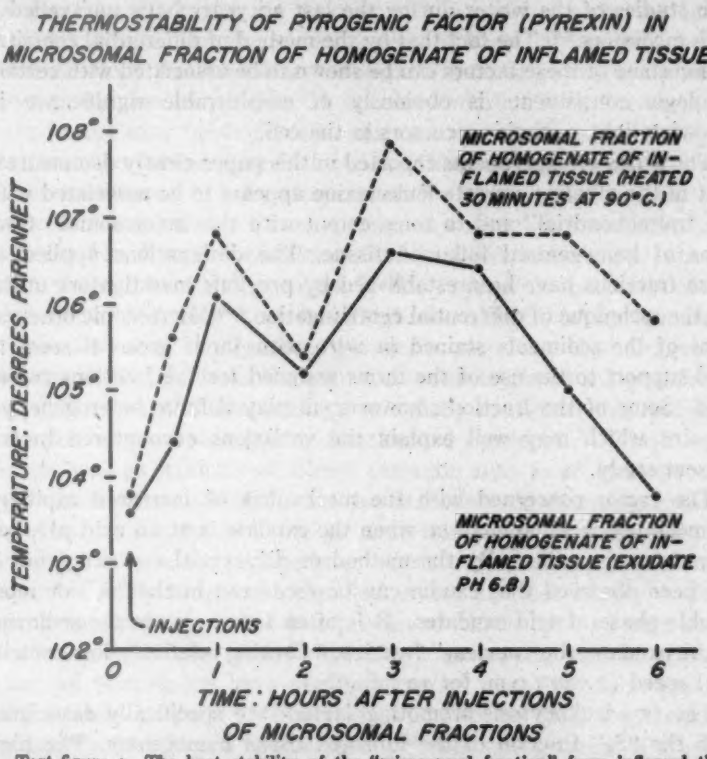
Comparison of Pyrogenic Activity of Various Fractions of Inflamed Tissue Homogenates with Those of an Acid Exudate (pH 6.7)

No. of rabbits	Maximum increase in temperature (°F.) within 2 to 6 hours								
		Nuclear fraction		S_2 fraction		Mitochondrial fraction		Microsomal fraction	
	Exudate	Exudate	Homogenate	Exudate	Homogenate	Exudate	Homogenate	Exudate	Homogenate
27	3.9	3.9	4.0	1.8	0.9	3.5	4.0	0.7	1.6
		3.7	3.8	2.4	0.4	3.4	1.6	1.8	1.1
							2.2		
	1.7	0.7	2.9	−0.3	2.1	−0.1	2.5	3.6	3.0
Average	2.8	2.8	3.6	1.3	1.1	2.3	2.6	2.0	1.9
	Increase or decrease in temperature with heated fractions								
45	0.6*	0.2	−0.2	−0.2*	0.6	−0.4	−0.8	0.5	0
	0.8*	0.2	0.2	0.1*		−0.7	−0.4	1.2	0.7
	2.0*	0.4	0.2	0.4*		−0.6	2.0		1.3
	2.5*	1.2	1.0	0.6*			2.2		
		2.8	1.8	0.8*			2.6		
		3.2		2.0*					
				2.4*					
	1.4*	0.2	2.9	0*	2.4	0	2.2	2.4	4.0
Average	1.5	1.2	1.0	0.8	1.5	−0.43	1.3	1.4	1.5

* Upon heating, these fractions formed a firm coagulum. This was broken mechanically and suspended in physiologic saline solution.

The fractions derived from acid exudates were heated to 90° C. for approximately 30 minutes in order to determine heat stability. As a consequence, some of the fractions tended to clot. Under such circumstances the coagulum was mechanically broken and suspended in physiologic saline solution. The saline suspension (0.5 ml.) was injected intravenously into test rabbits, and temperature was recorded at intervals for 6 hours. Considerable variation was encountered. The use of the suspended coagulum as well as the heterogeneity of some of the fractions might perhaps account for these variations. The data are assembled in Table VII. The "mitochondrial" fraction of exudates was completely inactivated by the heating procedure. This, however, failed

to occur with the same consistency in the case of the other fractions. With the whole acid exudate, the "nuclear," and apparently the "S_2" fractions, pyrogenic activity was only partly reduced. On the other hand, the "S_2" fraction of the tissue homogenate and the "microsomal" fraction seemed to be essentially unaffected by heating. At times pyrogenic activity actually appeared to be enhanced by heating. This was illustrated by the "microsomal" fraction of the tissue homogenate at pH 6.8 (Text-figure 3). Since pyrexin is heat stable[21,22] and is primarily



Text-figure 3. The heat stability of the "microsomal fraction" from inflamed tissue homogenate with exudate at acid pH (6.8).

recovered from an acid exudate, it is not surprising to encounter this heat stable factor in an acid exudate, and also in some of the fractions derived from it by differential centrifugation. The failure of heating to inactivate some fractions of inflamed tissue homogenates (Table VII; Text-fig. 3) suggests the existence of two pyrogenic factors, namely a heat labile one and a heat stable one. The characteristics of the latter are consistent with those of pyrexin.[2,21,22]

Discussion

The technique of differential centrifugation has its limitations. As Brachet and others have aptly demonstrated, it is difficult to make a sharp separation between the various cellular constituents.[26] Nevertheless, the method yields some topographic information in regard to the loci of significant biochemical components in cell organization. The injured cell in inflammation liberates into the exudate various mediators concerned with the diversified manifestations of inflammation. The studies of the writer during the last 20 years have unravelled 9 such mediators.[1,2] The fact that by the method of differential centrifugation some of these factors can be shown to be associated with certain cytologic constituents is obviously of considerable significance in throwing light on their precursors in the cell.

The series of experiments reported in this paper clearly demonstrate that in the alkaline exudate leukotaxine appears to be associated with the "mitochondrial" and to some extent with the "microsomal" fractions of homogenized inflamed tissue. The designations applied to these fractions have been established by previous investigators utilizing the technique of differential centrifugation.[3,4] Microscopic observations of the sediments stained *in vitro* with Janus green B seem to lend support to the use of the terms assigned for the fractions recovered. Some of the fractions, however, display definite heterogeneity,[4] a point which may well explain the variations encountered in the present study.

The factor concerned with the mechanism of increased capillary permeability in inflammation, when the exudate is at an acid pH, has been termed exudin.[7] By the method of differential centrifugation it has been observed that exudin can be recovered in the "S_1" or most soluble phase of acid exudates. It is often found also in the sediment of the exudate, the "nuclear" fraction, following relatively low centrifugal speed (2,000 r.p.m. for 10 minutes).

The two leukocytosis-promoting factors are specifically associated with the "S_2" fraction of the inflamed tissue homogenate. The high degree of solubility of the "S_2" fraction would suggest that the factors concerned with the mechanism of leukocytosis in inflammation are associated with submicroscopic cellular structures.

Pyrogenic activity in inflammation was first shown in 1944 to be referable to pyrexin, a chemical substance in the exudate.[21,22] This mediator has been shown to be heat stable and to be associated with the euglobulin fraction of exudates which are primarily acid.[2,21,22] Moreover, the relationship of leukopenia and leukocytosis to crude pyrexin has been shown to be due respectively to a leukopenic factor[20]

and to a thermostable leukocytosis-promoting factor,[18,19] both of which can be dissociated from pyrexin.[2,18-20] The writer has been able to demonstrate that bacterial pyrogens injected intravenously induce in rabbits the formation of a pyrogenic complex localized in the euglobulin fraction of blood serum.[29] Since the bacterial pyrogens may, in addition to hyperpyrexia, also induce leukopenia and leukocytosis, and since pyrexin is localized in the euglobulin of exudates, it is suspected that bacterial pyrogens induce a pyrexin-like substance that is likewise localized in the euglobulin fraction. This would explain the latent period which follows the administration of bacterial pyrogens before fever develops. Such a latent period fails to occur following the intravenous injection of pyrexin. A definite and significant increment in temperature may be detected as early as 15 minutes after the introduction of an active pyrexin fraction. Crystallized pyrexin also provokes an increase in body temperature.[28] The fact that pyrexin fails to cause the formation of any pyrogenic substance when introduced into the blood stream constitutes another difference in its behavior from that of bacterial pyrogens.[29] The pyrogenic property of the blood serum following intravenous injection of bacterial pyrogens may well reflect the "endogenous pyrogen" postulated by Grant and Whalen.[30] The studies of the writer have substantiated this postulate,[29] and Petersdorf and Bennett have essentially confirmed this finding in dogs without, however, having fractionated the serum.[31] These workers, as well as Atkins and Wood, maintain that fever accompanying inflammation is attributable to a thermolabile endogenous pyrogen.[24,25] It would be highly desirable to undertake chemical extraction and purification in order to determine whether the labile pyrogenic substance is really a separate chemical entity and not merely a stable pyrogenic substance linked to a protein which, by such an association, would render it heat labile. As far as I am aware, such attempts at chemical purification have not been carried out. The claim that the thermolabile pyrogen is liberated primarily by polymorphonuclear leukocytes is still open to some doubt.[32] The pyrogenicity of granulocytopenic exudates and those in patients with leukopenia is certainly not adequately explained by the assumption that the pyrogen is derived from neutrophils.[24,32] It would be far simpler at this stage to postulate the liberation of such an endogenous pyrogen from all injured cells at the site of inflammation, rather than to assign this property specifically to neutrophils. I have taken this view in respect to the origin of pyrexin and of other chemical mediators liberated at the site of inflammation.[1,2,21,22] A recent comment by King and Wood on my failure to demonstrate tolerance to pyrexin when sufficiently large

doses were administered to rabbits overlooks the real issue.[23] The criticism was originally raised by Bennett and Beeson[24] and concerned the adequacy of pyrexin to explain the pathogenesis of fever. This was based on their observations that small doses of pyrexin induced tolerance and therefore could not very well explain the mechanism of fever accompanying inflammation. This interpretation was shown by the writer to be erroneous, for if the concentrations of pyrexin were of the same magnitude as its concentration in exudate, no tolerance occurred following repeated injections of it.[2,29]

Nevertheless, the observations reported in the present communication suggest strongly that there are two pyrogenic factors in inflamed tissue and exudates. One of these was found in association with several cytologic constituents of injured cells. It was heat stable, and its characteristics were consistent with those of pyrexin. The other factor, found in the "S_2" fraction of alkaline exudates and also present in the "mitochondrial" fractions, appeared to be thermolabile and may possibly prove to be similar to the labile factor described by Bennett and Beeson.[24] The presence of two factors to explain the mechanism of fever accompanying inflammation is perfectly feasible. In earlier studies two separate factors were shown to account for a single manifestation of inflammation during the progression of the inflammatory reaction from an alkaline to an acid stage.[2,7,34] In this way, despite local changes, systemic homeostasis was maintained throughout the duration of acute inflammation.[7,34] A similar state of affairs has been demonstrated in the development of leukocytosis (LPF and thermostable leukocytosis factor), leukopenia (leukopenin and leukopenic factor), and increased capillary permeability (leukotaxine and exudin).[2,34]

Summary and Conclusions

Utilization of the technique of differential centrifugation on exudative material and on the homogenates of inflamed tissue indicated that the chemical mediators responsible for many of the biologic manifestations of inflammation are associated with some of the cellular constituents as follows:

Leukotaxine is found primarily in the "mitochondrial" and "microsomal" fractions of alkaline exudate, and in the corresponding fractions of inflamed tissue homogenate when the exudate is at an alkaline pH.

Exudin seems to be present practically only in the "S_1" and "nuclear" fractions of acid exudates.

Two leukocytosis-promoting factors are recovered in the "S_2" fraction of inflamed tissue homogenates.

The heat stable pyrogenic substance, pyrexin, is present in the "mitchondrial" fraction of alkaline exudates and in the corresponding "mitochondrial" fraction of inflamed tissue homogenate. Pyrexin tends to be present also in the "mitochondrial" and the "microsomal" fractions of inflamed tissue when the corresponding exudate is at an acid pH. This tendency does not, however, hold true in all experiments.

There is also a thermolabile pyrogenic factor in the "S_2" fraction of alkaline exudate and to a slight extent in "mitochondrial" fractions of alkaline exudates and in the corresponding homogenate of inflamed tissue. This labile factor is also demonstrable in the "nuclear" fractions of acid exudates and inflamed tissue homogenate. It is also found in the "mitochondrial" fraction of acid exudates.

The presence and the liberation by injured cells of two pyrogenic factors, a thermolabile and a thermostable factor (pyrexin), explains in reasonable manner the mechanism of fever during the course of acute inflammation. There is maintenance of systemic homeostasis despite the progressive local changes in hydrogen-ion concentration at the site of inflammation.

The observation that chemical mediators can be recovered from exudates in association with some of the cellular constituents of inflamed tissue is of significance in regard to a further understanding of the cytologic precursors of these biologically active substances.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. The accumulation of trypan blue from the circulating blood in cutaneous areas of the abdomen of a rabbit treated as follows: (1) 0.5 ml. of S_1 fraction from an acid exudate (pH 6.8) plus 0.25 ml. physiologic saline; (2) 0.5 ml. of same S_2 fraction as in area "1" plus 5 units of ACTH in 0.25 ml. of its vehicle; (3) 0.5 ml. of same S_2 fraction as in area "1" plus 5 mg. cortisone acetate in 0.1 ml. of its vehicle; (4) 0.5 ml. "nuclear fraction" derived from acid exudate (pH 6.8) plus 0.25 ml. saline; (5) 0.5 ml. "nuclear fraction" as used in area "4" plus 5 units of ACTH in 0.25 ml. of its vehicle; (6) 0.5 ml. of same "nuclear fraction" plus 5 mg. cortisone acetate in 0.1 ml. of its vehicle; (7) 0.5 ml. physiologic saline; (8) 0.25 ml. of vehicle containing 5 units ACTH. ACTH represses the increased capillary permeability induced by the S_1 and the "nuclear" fractions of an acid exudate. Cortisone fails to manifest such suppressive effect. Photograph taken about one hour after injections of fractions into the skin.

FIG. 2. Rabbit 29-85A. Cutaneous area of the abdominal skin 1 hour and 17 minutes after the introduction of 0.5 ml. of a mixture (0.5 ml. of "S_1" fraction derived from a canine acid exudate, pH 6.2, and 0.1 ml. saline). At this point the fraction failed to induce any appreciable leukocytic migration into the tissue spaces. Hematoxylin and eosin stain. × 255.

FIG. 3. Rabbit 44-58A. Skin of rabbit 56 minutes after the injection of the "mitochondrial" fraction of inflamed tissue from the pleural cavity of a dog; exudate, pH 7.45. There is both increased capillary permeability and local migration of leukocytes. Hematoxylin and eosin stain. × 255.

FIG. 4. Rabbit 44-58B. With 10 mg. cortisone added to the "mitochondrial" fraction, the increase in capillary permeability and leukocytic migration produced by the fraction fails to appear. Hematoxylin and eosin stain. × 255.

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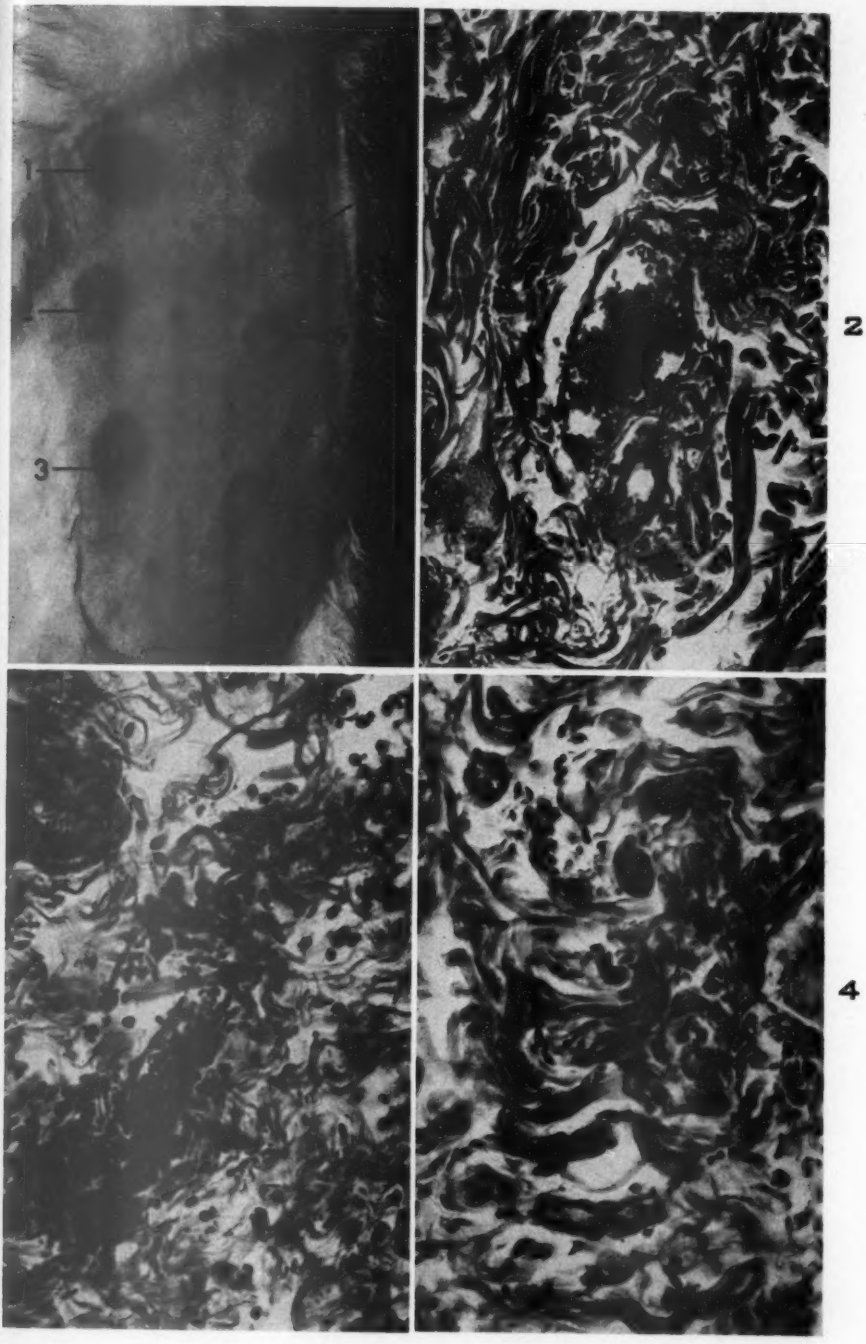
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CYTOPATHOLOGY OF HUMAN ENTERIC VIRUSES IN TISSUE CULTURE*

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The present study was undertaken to compare the cytopathogenic effects of human enteric viruses (poliovirus, Coxsackie virus, ECHO virus) in monkey kidney cultures in fixed and stained preparations. Enders,[1] in a review of the cytopathology of viruses, grouped viruses according to their reactions in tissue cultures and placed poliovirus and Coxsackie virus in one group, which was designated as producing cellular degeneration but no inclusion bodies. Buckley,[2] in preliminary studies, reported that the cytopathogenic effects of poliovirus types I, II, III; Coxsackie virus type B; and several ECHO viruses in monkey kidney tissue cultures appeared to be similar. Bernkopf and Rosin[3] recently compared the effects of the 3 human enteric virus groups in human amnionic tissue cultures.

The cytopathogenic effects of poliovirus in monkey kidney and HeLa cell cultures have been studied in detail. Some major changes described included distortion of nuclei, granularity of cytoplasm, and rounding and disintegration of cells.[1-4] The appearance of a cytoplasmic mass in infected cells has also been described.[5-9] Dunnebacke,[10] and Reissig, Howes and Melnick[8] considered nuclear alteration to be the most conspicuous feature, although both described the presence of the cytoplasmic mass. Reissig and co-workers[8] observed eosinophilic intranuclear inclusions in monkey kidney cultures and compared these findings with those of Sabin and Ward[11] and Bodian[12] who reported nuclear inclusions in the motor neurons of monkeys infected with poliovirus. Beale, Stevens, Davis, Stackiw and Rhodes[9] also mentioned the rare occurrence of eosinophilic granules or inclusions in the nuclei of cells which contained cytoplasmic masses. Buckley[2] and Reissig and co-workers[8] recorded the observation of basophilic cytoplasmic granules.

In the present investigation, in order to study the specificity of the cytologic alterations occurring in monkey kidney cell cultures infected with human enteric viruses, the development and subsequent degeneration of uninfected cultures were examined under physiologic as well as under unsuitable nutritional and physical conditions. The changes seen

* Aided in part by a grant from The National Foundation for Infantile Paralysis.
Received for publication, February 1, 1958.
† Markle Scholar in Medical Science.

under these conditions were compared with those accompanying enteric virus infection using poliovirus type I as a standard. Histochemical studies of cultures infected with poliovirus type I were also performed in an attempt to correlate cytochemical information with the structural changes observed.

Materials and Methods

Tissue Cultures

Monkey kidney monolayer tissue cultures were prepared by the trypsin-dispersal technique of Youngner[13] as modified by Melnick.[14] Cover slip cultures were prepared by placing specially cut 7 by 40 mm. cover slips in 16 by 125 mm. test tubes. Two ml. of cell suspension containing 350,000 to 400,000 cells per ml. were added; the tubes were stoppered and incubated stationary at a 5° slope in conventional tissue culture racks. The cultures were incubated at 36° C. and were exchanged with fresh medium after 3 to 4 days' incubation. The monolayer was complete and the cultures were ready for use 2 to 3 days after feeding.

Although detailed studies of cover slip cultures could not be made in the test tube, the gross structure of the cells and the condition of the monolayer could be readily established. Cells infected with virus could be identified by their increased refractility and rounded shapes. During the experiments, conventional test tube cultures were also employed as controls to facilitate early detection of virus and to permit better scrutiny of the cultures under the microscope.

The cells were implanted and fed with a growth medium consisting of 0.5 per cent lactalbumin hydrolysate, 2 per cent calf serum in a base of Hanks's balanced salt solution. For virus study, the growth medium was replaced at the time of inoculation with 1.8 ml. of maintenance medium which substituted Earle's saline for Hanks's balanced salt solution (BSS). The two media differed in that the Earle's maintenance medium, containing more $NaHCO_3$, maintained a pH of 7.0 or greater throughout the experiment, while the poorly buffered growth medium attained a pH of 6.6 to 6.8 within 48 hours. It has been shown that an acid pH is less favorable for the growth of most enteric viruses.[15]

Viruses

Poliovirus type I (Mahoney), type II MEF_1, and type III (Saukett) were obtained from Connaught Medical Research Laboratories, Toronto, Canada. Coxsackie viruses B1 through B5 and A9 were obtained through the courtesy of Dr. Gilbert Dalldorf, New York State

Department of Health, Albany, New York. ECHO virus prototypes, 1 to 14, were secured from members of the Committee on ECHO Viruses.[16] Cultures were inoculated with 0.2 ml. of undiluted virus suspension. All virus pools were prepared in monkey kidney tissue cultures. Titers of the viruses ranged from 4.0 to 6.5 log TCD per 0.1 ml., depending on the virus group and type.

Histologic Technique

Prior to fixation and staining, the supernatant fluid of the cover slip cultures was removed and discarded. The handling of infected culture fluids was facilitated by the use of a 30 ml. Luer-Lok syringe fitted with a stylet 4 inches long. The cultures were rinsed twice with warm BSS. During the second rinse, the cultures were allowed to stand 20 minutes at room temperature.

During the course of the study, many stains and fixatives and several histochemical techniques were utilized. The stains most frequently used were the May-Gruenwald-Giemsa stain[17] with cultures fixed in methyl alcohol; hematoxylin and eosin (H and E) stain with cultures fixed in Bouin's fluid; and the azure-eosin stain, as described by Lillie,[18] following fixation in absolute alcohol. Each of these stains demonstrated certain cytologic features better than others, but the H and E stain as modified by Reissig and co-workers[8] proved to be the most suitable method since it demonstrated most of the alterations characterizing the effect of the viruses. Cultures to be stained by this method were fixed in Zenker's solution for one hour. Unless otherwise indicated, the descriptions of the lesions were made on cultures stained by this method.[8]

Other stains, such as the Mann stain,[18] were used to supplement the H and E stain. The Marchi method for fat[18] and the Cowdry stain for mitochondria[18] were also used. Histochemical methods included the Feulgen reaction for DNA,[18] the Bauer technique for glycogen,[18] and the reaction for RNA, using the methyl-green-pyronin staining method with ribonuclease digestion as described by Pearse.[19] The fixatives used for these techniques were those recommended by the respective authors.

The staining reaction varied from culture to culture, and the elucidation of the pathologic changes was difficult to interpret at times because of this variation. In spite of the fact that each cover slip was handled in as nearly the same manner as possible, very minor variations in technique caused great differences in staining reaction.

Differential counts were made, using the oil immersion objective. At

each time interval after infection, 1,000 cells per cover slip were counted. Uninfected control cultures were counted simultaneously. Measurements of structures were made with the Spencer micrometer under the oil immersion objective.

Experimental Procedure

Monkey kidney cultures were inoculated with poliovirus type I. Infected cultures and controls were rinsed, fixed, and stained just after inoculation, at hourly intervals between 2 and 8 hours, and at 10, 13, and 25 hours after inoculation. Cultures were also inoculated with poliovirus types II and III and Coxsackie and ECHO viruses. Cover slips were processed when the cultures showed all the microscopic alterations seen with poliovirus type I. The times at which these tubes were processed varied from 7 to 25 hours after inoculation, and the lesions varied from initial to well advanced stages of infection.

In uninfected cultures the development of monkey kidney monolayer and its subsequent degeneration under different nutritional and temperature conditions were studied as follows: (a) after implantation until exchange with growth medium; (b) developing cultures without change of medium; (c) developing cultures exchanged with growth medium on the fourth day; (d) developing cultures exchanged with maintenance medium on the fourth day; (e) complete monolayer cultures exchanged with maintenance medium and incubated at 45 to 50° C. and at 5° C.; (f) complete monolayer cultures exchanged with BSS and incubated at 36° C.

Results

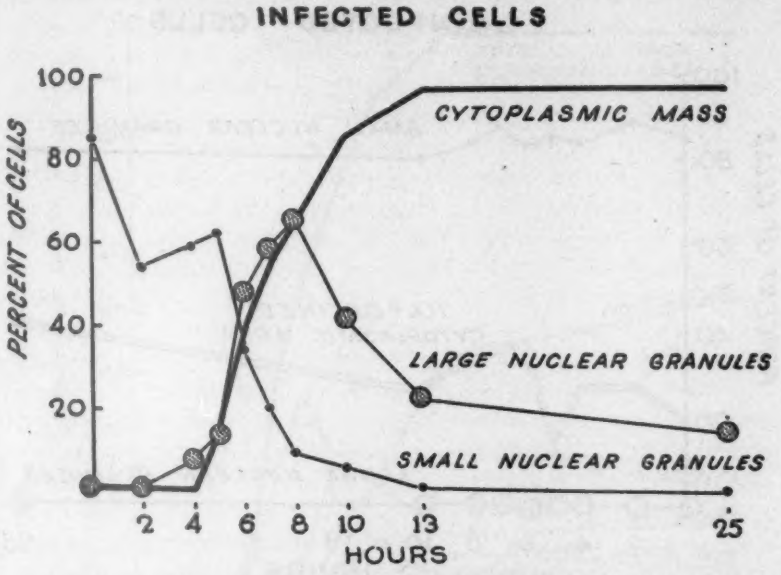
Infection with Poliovirus Type I

Nuclear Changes. A significant nuclear alteration appeared 4 hours after inoculation. This consisted of an increase in the number of nuclei containing large eosinophilic granules measuring 1 μ or more in greatest diameter (0.2 per cent at 2 hours to 7.3 per cent at 4 hours) (Text-fig. 1; Fig. 2). The number of nuclei containing these large granules increased up to 8 hours (65 per cent), after which there was a fairly sharp and steady decrease to 13 hours. After 13 hours, the decrease was more gradual.

There were also weakly eosinophilic intranuclear granules measuring 1 μ or less. These small granules were present in many nuclei up to and including 5 hours, but thereafter showed a steady decrease as the nuclei with larger granules increased (Text-fig. 1). Nuclear granules were seen also in the uninfected control cultures, but the majority of these were of the small, weakly eosinophilic variety. Only a few nuclei

contained granules measuring more than 1 μ (0.0 to 0.6 per cent) (Text-fig. 2).

In both infected and control cultures, the nuclear granules, large or small, were variable in shape, size and number. However, in infected cultures, the large granules were usually round and more eosinophilic.

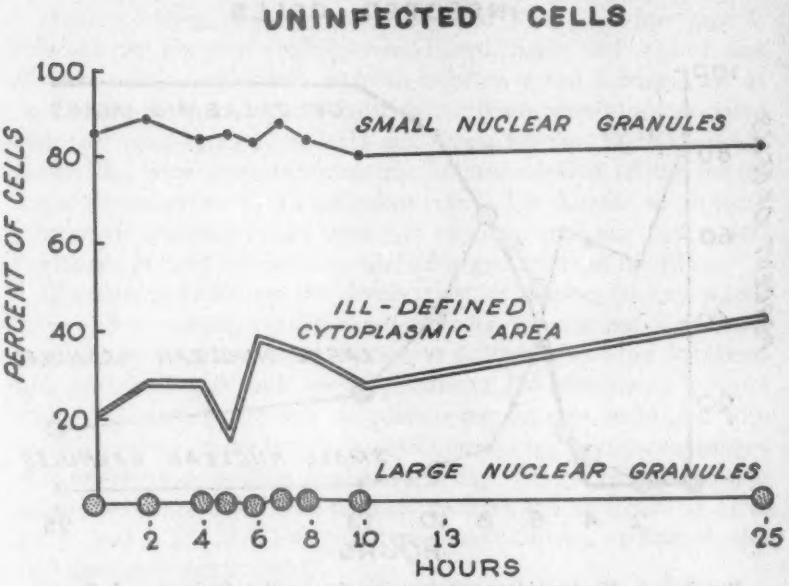


Text-figure 1. Monkey kidney monolayer inoculated with poliovirus type I. Quantitative analysis of cells containing eosinophilic cytoplasmic masses, nuclei with large eosinophilic granules, and nuclei with small, weakly eosinophilic granules.

The number of granules varied from 1 to 10 per nucleus and measured from less than 1 μ to 3 μ in diameter. It was noted that as infection progressed, the number of granules in a given nucleus decreased. In the later stages, only one or two per nucleus were present, and these were more rounded and deeply eosinophilic. In the very late stages, the nuclei became pyknotic and no granules were seen.

Cytoplasmic Changes. Alterations in the cytoplasm began at 5 hours after inoculation. At this time, about 13 per cent of the cells showed a single, well defined, eosinophilic cytoplasmic mass adjacent to the nucleus (Text-fig. 1; Fig. 3). The mass frequently displaced the nucleus to one side of the cell, and in a few cells the mass indented the nucleus. The number of nuclei with such indentations increased as infection progressed. The cytoplasmic mass varied from 15 to 25 μ in diameter and was fairly homogeneously stained with H and E. At 5

hours, about 9.6 per cent of the cells containing cytoplasmic masses also showed large nuclear granules. As infection progressed, the number of cells with cytoplasmic masses increased steadily until approximately 13 hours, after which the increase was very slight (Text-fig. 1). Up to and including 8 hours, the number of cells with cytoplasmic



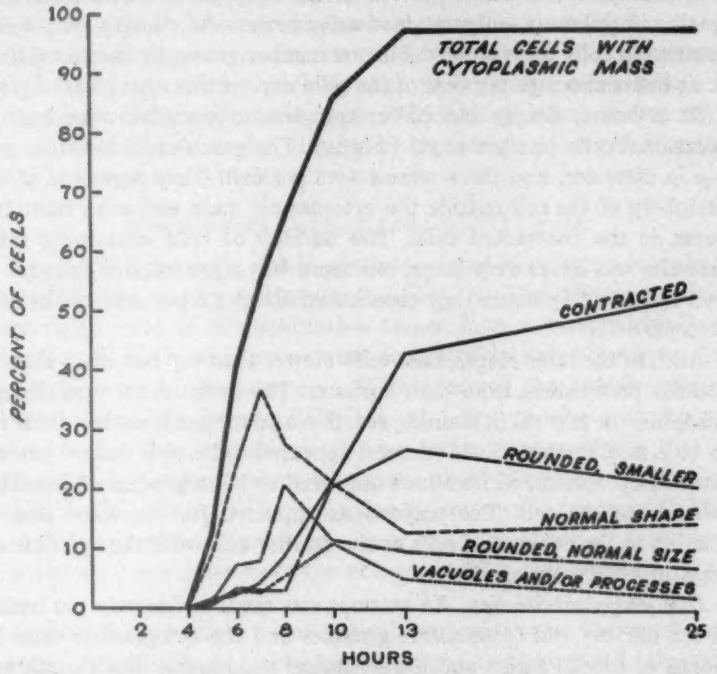
Text-figure 2. Uninfected monkey kidney monolayer. Quantitative analysis of cells with small, weakly eosinophilic nuclear granules, large eosinophilic nuclear granules, and ill-defined eosinophilic cytoplasmic zones.

masses and large nuclear granules increased. After 8 hours, cells containing cytoplasmic masses, but no nuclear granules, continued to increase.

In all uninfected control cultures, an ill-defined, slightly eosinophilic area was seen in the cytoplasm of many cells in about the same position as the well defined cytoplasmic mass in infected cultures. This area was also observed in the uninvolved cells in infected cultures. The percentage of cells demonstrating this feature was difficult to determine accurately because its appearance depended on the staining reaction of the cultures, and this was distinctly variable (Text-fig. 2). The weakly eosinophilic area in the cytoplasm of uninfected cells will be referred to as the "cytoplasmic area," and the expression "cytoplasmic mass" will be used to indicate the alteration in infected cells.

Other alterations occurring in the cells coincidentally with those

described included: rounding of cells; the appearance of vacuoles in the periphery of the cytoplasm; the appearance of cytoplasmic processes; contraction of cells; and the appearance of basophilic cytoplasmic granules. These changes were found in cells containing eosinophilic cytoplasmic masses. Nuclear granules were present in



Text-figure 3. Monkey kidney monolayer inoculated with poliovirus type I. Quantitative analysis of the morphologic variations in cells which contained eosinophilic cytoplasmic masses.

some and absent in others. Text-figure 3 constitutes a quantitative analysis of these morphologic variations in cells containing cytoplasmic masses.

Aside from the large nuclear granules and the cytoplasmic mass, rounding of cells was the next most common change observed. The appearance of vacuoles and cytoplasmic processes seemed to represent an intermediate stage, following the rounding of cells and preceding their decrease in size and the indentation of their nuclei (Text-fig. 3). As infection progressed, the cells became smaller, more rounded, showed a thin peripheral layer of cytoplasm, and exhibited increased prominence of the eosinophilic cytoplasmic mass. The latter, in many

cells, caused indentation of the nucleus. The number of cells showing this feature reached a maximum at about 13 hours (26.8 per cent).

In the very late stages of infection, the cells appeared to be contracted, measuring from 9 to 14 μ in diameter. These were round cells which had decreased markedly in size and in which the cytoplasmic mass occupied the major portion of the cytoplasm. The nuclei were small and pyknotic and contained no granules. At 5 hours, only a few contracted cells were present, but the number gradually increased until at 25 hours about 52 per cent of the cells were of this type (Text-fig. 3).

At 8 hours, deeply basophilic cytoplasmic granules were seen in occasional cells (0.1 per cent) (Fig. 4). The granules varied from 1 to 4 μ in diameter, and there were 1 to 6 per cell. They appeared at the periphery of the cell outside the cytoplasmic mass and were most frequent in the contracted cells. The number of cells containing these granules was never very large, but there was a progressive increase so that at 13 and 25 hours they constituted about 1.6 per cent of the cells present.

Also, in the later stages, rare cells (fewer than 0.5 per cent) showed nodular protrusions from their surfaces. The protrusions were slightly basophilic or neutral in staining reaction and ranged from less than 1 μ to 10 μ in diameter. Some of them appeared to be well defined projections of cytoplasm, while others appeared to be in process of breaking away from the cell. The projections appeared for the most part in relation to the contracted cells or the smaller cells with the cytoplasmic mass indenting the nucleus.

Histochemical Studies. An attempt was made to identify the nature of the nuclear and cytoplasmic granules and the cytoplasmic mass by means of special stains and histochemical techniques. Both small and large nuclear granules gave negative reactions for glycogen, lipid, DNA and RNA. When the Mann stain was used, the granules exhibited eosinophilic staining; the nucleoli were basophilic.

Glycogen was not regularly demonstrable in the cytoplasmic mass, the results varying from experiment to experiment. In two of the experiments, the amount of glycogen in the cells of infected cultures was identical with that in the uninfected cultures. The glycogen was usually seen at the periphery of the cytoplasm outside the mass. In one of the experiments, there was more glycogen present in the infected cultures. A great deal of it was concentrated in the cytoplasmic mass, although some droplets were diffusely distributed throughout the cell. In the uninfected cultures, glycogen was more diffuse in arrangement but slightly more concentrated in the eosinophilic area.

In some of the infected cultures, lipid droplets were grouped about

the nucleus, while in others they were diffusely distributed. The cytoplasmic mass gave negative reactions for DNA. With the Mann stain, the cytoplasmic mass was eosinophilic, and with azure-eosin it stained a pink color. Cytoplasmic granules gave negative reactions for glycogen, lipid, DNA and RNA. They were eosinophilic following the use of Mann's stain, and stained a deep blue with the azure-eosin method. The latter stain was the best for demonstrating these granules.

Mitochondria appeared concentrated in the area of the cytoplasmic mass in infected cultures, while in controls they were more diffuse in arrangement but slightly more numerous in the cytoplasmic area adjacent to the nucleus. Beale[9] also found concentration of mitochondria in the region of the cytoplasmic mass.

Infection with Coxsackie and ECHO Viruses

No essential cytologic differences were noted in cultures inoculated with any of the enteric viruses except ECHO 10. The differences which were noted were of a quantitative rather than a morphologic type when the other enteric viruses were compared to poliovirus type I at the same stage of development. With some of the enteric viruses, namely, Coxsackie B4 and ECHO 4, 8, 9 and 14, the infection appeared to be more "explosive" with a greater number of the affected cells appearing immediately as the small, contracted type. With Coxsackie B2 through B5, ECHO 3, 4 and 14, more cells appeared to have basophilic cytoplasmic granules. Since only one strain of a serotype was tested, these differences are not necessarily characteristic of the type.

Certain factors made quantitative interpretation of the results uncertain. It was difficult to harvest the preparations at exactly the same stage of cell involvement, and the titers of the viruses varied. Despite this, there were apparent differences in the rate of development of the lesions except in cultures inoculated with ECHO 7 and 9 viruses. The alterations here proceeded at the same rate as those in cultures infected with poliovirus type I. The other enteric viruses took from 2 to 25 hours longer than poliovirus type I to develop the changes.

ECHO 10 caused changes which distinguished it from the others (Fig. 5). Many cells contained homogeneous, deeply eosinophilic masses in the cytoplasm. These were irregular, dense, and appeared either adjacent to the nucleus or surrounded it, but did not indent or displace it. In some cells, the masses were broken into globules which were spread fairly diffusely in the cytoplasm. In others, the eosinophilic material appeared as thin strands running through the cytoplasm. Scattered throughout the cultures were cells which re-

sembled keratinized squamous cells. These increased in number in cultures which were infected for longer periods of time and were assumed to be late stages in the pathologic process.

Uninfected Cells

After Implantation Until Exchange with Growth Medium. On the first day after implantation with the monkey kidney cell suspension, there were groups of 5 to 6 small cells (10 μ, rarely 30 μ). Cytoplasm was somewhat vacuolated, and many cells contained eosinophilic debris in globules or masses of varying size. The nuclei stained poorly and contained no nuclear granules. Other cells, which appeared in process of degeneration, were small, round and deeply eosinophilic. No mitoses were seen in any of the cultures. As the cultures aged, the cell groups became larger, and the cells also increased in size (at 2 days, 10 to 40 μ; at 3 days, 10 to 50 μ). Small nuclear granules were seen in a few cells. The number of cells containing intracytoplasmic eosinophilic debris decreased as the cultures developed, and an increasing number of cells were seen to have ill-defined, faintly eosinophilic areas adjacent to their nuclei. The number of cells containing small nuclear granules was increased. Degenerating cells were decreased in number, and occasionally healthy cells contained a tiny engulfed eosinophilic cell in the cytoplasm (Fig. 6). Mitotic figures began to appear on the second day and increased in number each succeeding day.

Developing Cultures Without Change of Medium. The monolayer was complete between 5 and 6 days after implantation (Fig. 1). The cells varied in size and shape, the majority being about 50 μ in diameter. Cell outlines were well defined, and many contained an intracytoplasmic eosinophilic area adjacent to the nucleus. Some cells exhibited deeply eosinophilic debris in the cytoplasm; rare cells contained engulfed degenerating cells as described above. About 75 per cent of the nuclei revealed small eosinophilic granules. Many mitotic figures were seen, and the number increased up to 18 days after implantation. In thicker portions of the cultures, some cells were spindle-shaped and others were very small, round and eosinophilic. At 11 days, the arrangement of the cells was somewhat altered. There was clumping of the cells, with many small, round cells especially numerous in the clumped areas. The number of nuclei containing granules decreased to about 50 per cent. From 11 to 18 days, the cells appeared to decrease in number, with some showing cytoplasmic disintegration. The decrease in number of cells, the lysis of cells, and the increase in small round cells continued until the end of the experiments at 25 days. Eosinophilic areas were seen in the cytoplasm of healthy cells, and the

number of elements with small nuclear granules decreased until, at 25 days, only about 30 per cent of the cells contained the granules. Rare small round cells had a prominent eosinophilic cytoplasmic area, slightly resembling the cytoplasmic mass in virus infected cells. The rate of degeneration of cultures varied from experiment to experiment, with some degenerating in 15 days.

Cultures Exchanged with Growth Medium on the Fourth Day. These cultures resembled those of the preceding group without change of medium.

Cultures Exchanged with Maintenance Medium on the Fourth Day. These cultures appeared to mature and disintegrate later than was the case in the untreated group. With continued incubation, the cells became spindle-shaped and underwent lysis with only a few small round cells remaining.

Complete Monolayer Cultures Exchanged with Maintenance Medium and Incubated at 45° to 50° C. and 5° C. Abnormally high temperature completely dried the cells after as short an exposure as 5 hours. Cold produced interesting changes (Fig. 7). No alterations appeared until 25 hours after exposure of cultures at 5° C. At this time, the cells generally appeared slightly contracted and rounded; the number with nuclear granules decreased to about 50 per cent. At 5 days, many cells were rounded and contracted, and the cytoplasm appeared dense. Eosinophilic cytoplasmic areas, resembling the cytoplasmic masses observed in cells of virus infected cultures, were prominent; some appeared fibrillar. There were many small round cells. Twenty per cent of the cells had nuclear granules. At 7 days, the monolayer was fragmented; many cells were round and, as before, denser in appearance. There were many small round degenerating cells. A few small round cells had neutral staining or slightly basophilic protrusions extending from their surfaces. Eosinophilic masses were present and prominent in larger cells; many appeared to be fibrillar. The nuclei were not indented, and about 20 per cent contained granules.

The monolayer, as time went on, became more and more fragmented, and greater numbers of cells became rounded. Many contained a prominent, well defined, eosinophilic cytoplasmic mass which was sometimes fibrillar and did not indent the nucleus. The nuclei appeared to be of reduced size, and about 3 per cent contained granules. By the end of the experiment (21 days), the cells were reduced in number, and the majority were small and round with tiny pyknotic nuclei. The larger cells contained prominent eosinophilic cytoplasmic masses. Some cells showed neutral-staining surface protrusions. Two to 3 per cent of the nuclei contained granules.

TABLE I

Sequence of Cytologic Changes in Monkey Kidney Cells

Cellular changes	Uninfected	Infected with poliovirus type I						
Nuclear	Small, weakly eosinophilic granules	Large eosinophilic granules →	Large eosinophilic granules →	Large eosinophilic granules →	Reduced numbers of eosinophilic granules →	No granules; pyknotic nuclei	Complete degeneration	
					Indented nucleus			
Cytoplasmic	Eosinophilic area	Eosinophilic area →	Eosinophilic mass →	Eosinophilic mass →	Prominent eosinophilic mass →	Prominent eosinophilic mass		
					Basophilic granules →	Basophilic granules		
Size and shape	Polygonal or spindle-shape; normal size	Polygonal or spindle-shape; normal size →	Rounding; normal size →	Rounding; peripheral vacuoles or processes →	Decrease in size of rounded cells →	Contraction of cells		

Complete Monolayer Cultures Exchanged with BSS and Incubated at 36° C. These cultures, maintained in physiologic saline solution only, showed fragmentation of the monolayer and grouping of cells. The latter became spindle-shaped and showed progressive disintegration of cytoplasm. Some cells appeared shrunken and elongated. Eosinophilic areas in the cytoplasm were not prominent, and nuclei with nuclear granules decreased in number. By the tenth day, many cells were destroyed, and those remaining were small, elongated, and showed lysis of cytoplasm.

DISCUSSION

Qualitative and quantitative observations of the cytologic effects of poliovirus type I on cultures of monkey kidney cells indicated that a certain sequence of changes occurred. These are summarized in Table I. Other authors have described a similar progression of events. Lwoff, Dulbecco, Vogt and Lwoff[6] examined single cells under phase-contrast microscopy. Barski, Robineaux and Endo[5] studied the cyto-

pathogenic alterations of human fibroblasts under phase-contrast and bright-light microscopy, and Harding, Harding, McLimans and Rake[7] described the progression of events as seen in the living state as well as in fixed and stained preparations of HeLa cells. Beale and co-workers[9] recorded the progression of alterations in monkey kidney tissue cultures, using the bright-light microscope.

In the present study, the following pathologic features were prominent: eosinophilic nuclear granules, cytoplasmic mass formation, and basophilic cytoplasmic granules. Interest in the presence of eosinophilic nuclear granules was stimulated by the work of Reissig and co-workers[8] who described nuclear inclusions in monkey kidney cell cultures infected with poliovirus. In our own earlier studies, these granules or "inclusions" had been overlooked, and only after using the H and E staining method as described by Reissig could these or similar granules be detected. In the present investigation, eosinophilic nuclear granules were observed in uninfected as well as in infected cultures. It was felt, therefore, that the granules seen in the infected cells might represent only morphologic changes in pre-existing structures rather than newly formed structures initiated by viral infection. The presence of similar nuclear granules in uninfected human and hamster kidney cultures, prepared and stained by identical methods, appeared to make it less likely that the nuclear granules were related to latent virus infection in cultures of monkey kidney. However, the increase in eosinophilia and size of the granules, and the rounding of their outlines appeared to be specific reactions to infection with virus. The early appearance of large nuclear granules may indicate that the first effect of the virus is on the nucleus. The cytoplasmic mass persisted after the nuclear granules disappeared and the nucleus became pyknotic. The intracellular localization of poliovirus type II in mice was studied by Kaplan and Melnick,[20] employing the technique of cell fractionation and centrifugation. The presence of infective virus was demonstrated in the nucleus during the early phases of infection. Buckley,[21] utilizing the Coons fluorescent antibody technique, described fluorescent material in the nucleus as well as in the cytoplasm.

In our experiments, the eosinophilic cytoplasmic mass was also thought to represent an alteration in a pre-existing structure rather than a new formation initiated by virus. In uninfected cultures, there was an ill-defined eosinophilic area adjacent to the nucleus in many cells. The area and mass could not be specifically identified histochemically, but in some infected cultures, glycogen, fat, and the mitochondria appeared to be more concentrated in this area.

A feature which appeared to be specific for virus infection was the

presence of basophilic cytoplasmic granules in the later stages. These granules were not found in uninfected cultures. They could not be identified by histochemical techniques. Buckley[2] reported basophilia and basophilic granules in the cytoplasm stained by the Giemsa method. Using the fluorescent antibody technique, she correlated the distribution of the basophilic material with that of fluorescent material.

Bernkopf[3] recently described certain distinguishing pathologic changes in human amnionic tissue cultures infected with human enteric viruses. These were straight cytoplasmic processes, with sharp separation of ectoplasm and endoplasm, and the retention of polygonal or triangular configuration. Based upon these features, a differentiation between poliovirus, Coxsackie and ECHO virus infection was reported. In the present study, only ECHO 10 caused distinguishing cytologic alterations in the monkey kidney monolayer. The main differentiating features induced by the ECHO 10 virus were a more globular and dense cytoplasmic mass and a different type of cell disintegration in the end stage. These findings added to the evidence that the ECHO 10 virus was biologically different from other members of the ECHO group. ECHO 10 is infectious for suckling mice and chick embryos and is larger in size than the other ECHO viruses.[16,22] None of the other enteric viruses could be differentiated from each other by their cytologic effects in monkey kidney monolayer. The only major difference was the rate of development of the lesions.

In the uninfected monkey kidney cultures which were studied under physiologic as well as under unsuitable nutritional and temperature conditions, there were certain features which were similar to those observed in cultures infected with the enteric viruses. In a physiologic nutritional state, rare cells in culture showed an eosinophilic cytoplasmic area resembling the cytoplasmic mass seen in infected cells. These appeared only in the later stage of culture growth. Storage at low temperature produced changes more closely resembling those encountered in enteric virus infection. The cytoplasmic masses were fairly prominent although they appeared later and were fewer in number. Protrusions from the outer surfaces of the cells were also noted. Features which differentiated the lesions observed under these conditions from those appearing in infected cultures included the absence of large eosinophilic nuclear granules, the lack of indentation or displacement of the nucleus by the cytoplasmic mass, and the absence of basophilic cytoplasmic granules. Nuclear granules were present, but they were small, and there was a gradual reduction in their number with aging of the culture. Maintenance in BSS produced lysis of cells;

there was no similarity to the cytologic alterations induced by virus infection.

Certain features such as rounding of cells, the presence of cytoplasmic masses, and the existence of protrusions from the cells observed in tissue cultures inoculated with enteric viruses were duplicated by forms of nonviral degeneration. Our observations have led us to conclude, however, that the overall pattern and sequence of changes in viral infection were distinctive and easily distinguished in stained preparations.

Summary

A comparison of the cytologic effects of polioviruses and Coxsackie and ECHO viruses in monkey kidney tissue cultures revealed a remarkable similarity. Only ECHO 10 produced changes which differed significantly.

Certain structures in virus infected cultures, such as large nuclear granules and cytoplasmic masses, were of special interest. These were postulated to be pathologic changes in structures which pre-existed in uninfected cells. Basophilic cytoplasmic granules were not found in uninfected cultures, and it was thought that these were the only lesions which, at the present time, could be considered to be specifically induced by virus infection.

The rounding of cells and the appearance of cytoplasmic areas with eosinophilic staining, resembling the cytoplasmic masses seen in virus infected cells, were found in uninfected cultures under varying conditions. However, the cytopathogenetic pattern in infected cultures was distinctive and easily distinguished.

The nuclear granules, cytoplasmic masses and basophilic cytoplasmic granules were not identified histochemically.

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The guidance of Dr. Kornel L. Terplan, Department of Pathology, University of Buffalo School of Medicine, and the assistance of Miss Helen E. Sanderson and Miss Alicia Bukowski are gratefully acknowledged. Photographs of the slide preparations were taken by Mr. Melford E. Diedrick, Medical Illustrator, University of Buffalo School of Medicine.

[*Illustrations follow*]

LEGENDS FOR FIGURES

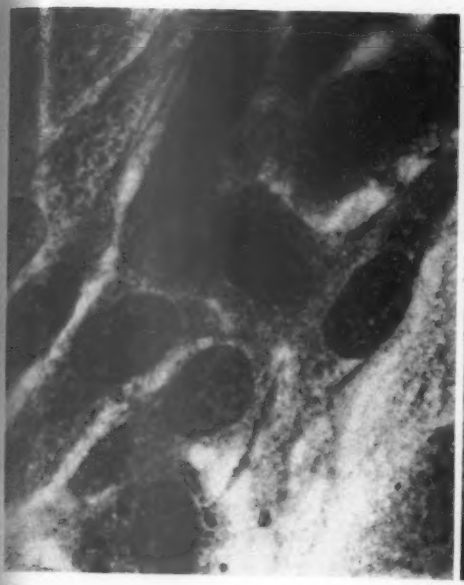
All sections stained with hematoxylin and eosin stain.

FIG. 1. Uninfected monkey kidney monolayer (6 days). Cytoplasmic areas (A) are seen adjacent to the nucleus. They are weakly eosinophilic in contrast to the remainder of the cytoplasm. Small eosinophilic nuclear granules contrast with the large basophilic nucleoli. × 1,200.

FIG. 2. Cell from a monkey kidney monolayer 4 hours after inoculation with poliovirus type I. Large nuclear granules are eosinophilic in contrast to the larger basophilic nucleoli. The enlargement of the cells as shown in Figures 2 and 3 did not appear to be characteristic of poliovirus infection. Enlarged cells occurred also in the uninfected control cultures. × 1,200.

FIG. 3. Cell from a monkey kidney monolayer 5 hours after inoculation with poliovirus type I. A well defined, eosinophilic cytoplasmic mass (M) is seen adjacent to the nucleus. There are also large nuclear granules. × 1,200.

FIG. 4. Cell from a monkey kidney monolayer 8 hours after inoculation with poliovirus type I showing peripheral basophilic cytoplasmic granules. × 1,200.



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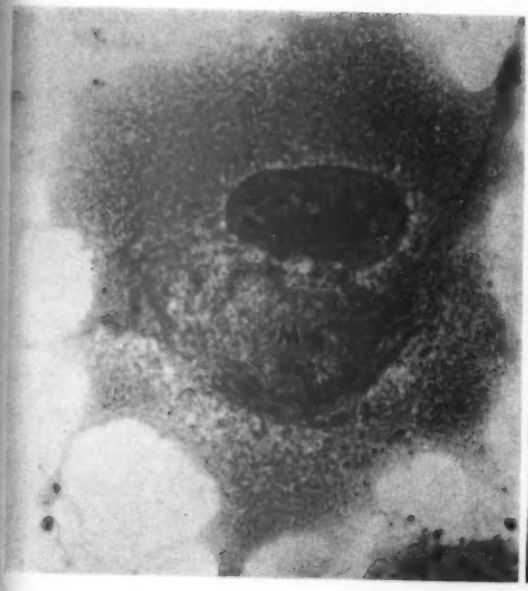

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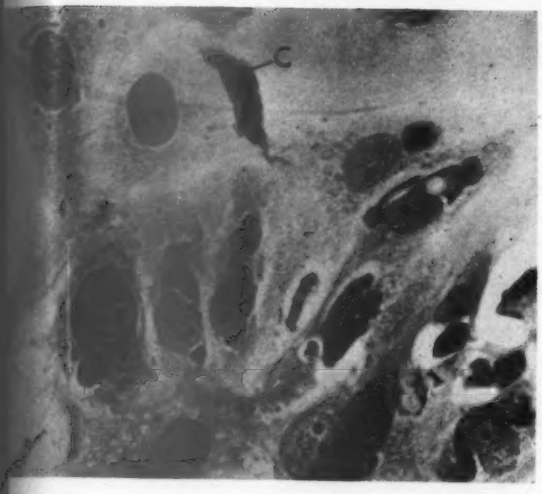
FIG. 5. Cells from a monkey kidney monolayer 75 hours after inoculation with ECHO 10 virus. Dense, globular, eosinophilic masses may be seen surrounding the nuclei. An elongated, degenerating cell, resembling a keratinized squamous cell, is also evident in the upper portion of the photograph (C). × 500.

FIG. 6. Cell from an uninfected monkey kidney monolayer 4 days after implantation. An engulfed cell is seen in the cytoplasm (D). × 1,200.

FIG. 7. Cells from monkey kidney monolayer incubated at 5° C. for 7 days. Some cells exhibit rounding and eosinophilic cytoplasmic zones. Others show protrusions from the surface (encircled cell). Protrusions of this type were seen occasionally in cultures infected with enteric viruses. × 500.



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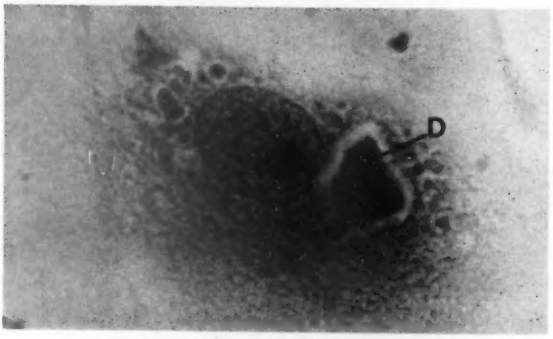

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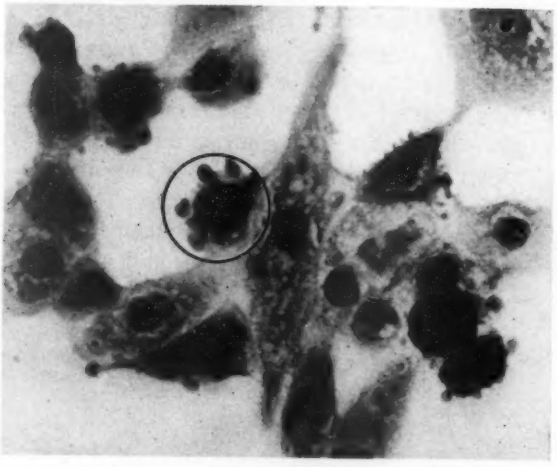

7

INHERITED (CONGENITAL) CATARACT IN THE DOG *

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This report deals with the various stages in the development of inherited lens opacity and associated ocular defects observed in purebred beagle puppies. The features of the primary lens defect serve as diagnostic means of distinguishing congenital cataract from other forms of similar lenticular opacity.

Materials and Methods

During a 4-year period, 1,129 purebred beagle puppies (233 litters) were whelped in our breeding colony. Of this number, one male (57E) evidenced cataract. The condition was first observed when he was 5 months old, and ophthalmoscopic examination revealed complete lens opacity in both eyes. Although this dog was totally blind, he was well adjusted to his environment and appeared in excellent physical condition. He was one of a litter of 3 males and 2 females; the other sibs had normal vision at 1½ years of age.

Beagle 57E was retained in our breeding colony and sired 5 litters from which 13 male and 12 female puppies were raised. All except 2 males (25C and 25D) were sacrificed by electrocution (110 v.) when they were between 41 and 60 days of age. Ante-mortem ophthalmoscopic examination had revealed varying degrees of cataract formation in 22 of the puppies.

Post-mortem examination was conducted immediately. Body and organ weights were obtained. The eyes were enucleated and placed in Bouin's fixative. After one hour in the fixative, a cap was carefully removed from each eye to allow penetration of the fixative into the inner portion of the eye. Thus, the normal size and curvature of the eye was retained. When fixation was complete (24 to 36 hours), the eyes were dehydrated, infiltrated with nitrocellulose, and sectioned. The sections, which were 6 to 15 μ thick, were transferred to slides directly from the knife. A total of 25 to 100 sections were cut in each eye through various planes of the lens. The following histologic stains were used: hematoxylin and eosin, Pollak's rapid trichrome stain,[1] 1 per cent toluidine blue O, and the Hotchkiss stain for polysaccharide.[2] Each skull was prepared for a detailed examination which included measurement of bones and foramens.

* This study was performed under A.E.C. Contract AT 11-1 GEN 10, "The Effects of X-Radiation on Longevity and Work Capacity in the Dog."
Received for publication, February 7, 1958.

Genetic Aspects of Inherited Cataract

Table I illustrates the genetic relationship of the dogs used in this study.

There was no evidence of cataract in the ancestry of dog 57E or in his 4 full sibs. Matings of 57E to 4 unrelated females and one sib

Table I

Cataractous Offspring of Male 57E

Mate no.	Litter	Offspring	Sex	Eye Condition*		Age (days) sacrificed
				Right	Left	
936	225	25A	F	Cataract (c)†	Cataract (c)	60
		25B	F	Cataract (c)	Cataract (c)	60
		25C	M	Normal	Normal	365
		25D	M	Normal	Normal	365
436	228	28A	F	Cataract (c)	Cataract (c)	43
		28B	F	Cataract (c)	Cataract (c)	43
		28C	F	Cataract (c)	Cataract (c)	43
		28D	M	Cataract (c)	Cataract (c)	43
		28E	M	Cataract (c)	Cataract (c)	43
732	229	29A	M	Cataract (c)	Cataract (c)	41
		29B	M	Cataract (c)	Cataract (c)	41
		29C	M	Cataract (p)	Cataract (p)	41
		29D	M	Cataract (c)	Cataract (c)	41
732	237	37A	F	Normal	Normal	50
		37B	F	Cataract (c)	Cataract (c)	50
		37C	F	Cataract (c)	Cataract (c)	50
		37D	M	Cataract (c)	Cataract (p)	50
		37E	M	Cataract (c)	Cataract (c)	50
		37F	M	Cataract (c)	Cataract (c)	50
		37G	M	Cataract (c)	Cataract (c)	50
640‡	238	38A	F	Cataract (c)	Cataract (c)	46
		38B	F	Normal	Cataract (p)	46
		38C	F	Cataract (c)	Cataract (c)	46
		38D	F	Cataract (c)	Cataract (c)	46
		38E	M	Cataract (c)	Cataract (c)	46

* Determined by histologic examination.
† (c) complete; (p) partial.
‡ Full sib to 57E.

produced 19 offspring with complete cataracts in both eyes; one offspring with complete cataract in the right eye and partial cataract in the left eye; one offspring with partial cataract in the left eye and a normal right eye, and 3 offspring with normal eyes.

One normal son from 57E was mated to the sib of 57E that had previously produced cataractous offspring when mated to 57E. The 4 males and 2 females whelped were all normal when examined at 2½ months of age. No further tests were made since all the pups were sacrificed for pathologic study.

It seems certain that the trait has a genetic basis since it was transmitted from parent to offspring through the male gamete. However, the high incidence of complete cataract was somewhat surprising. If the lenticular abnormality was caused by a dominant gene in 57E, the probability of so large a deviation (22:3) from the expected ratio in either direction is 0.0009. The trait apparently was not sex linked since normal and cataractous eyes were observed in both male and female offspring. Further speculation as to the manner of transmission does not seem advisable without additional genetic data.

Discussion

Cataract of genetic origin has been reported in several breeds of dogs. Westhues[3] described 40 cases among 1,200 mongrels. The condition was also reported in the German shepherd breed by Hippil,[4] and in pointers by Høst and Sveinson.[5] The lesion was observed in these during the second, third, and fourth years of life.

We have observed inherited cataract in both cocker spaniel and beagle breeds. It was recognized in cocker spaniels between 9 and 18 months of age and was first observed in beagle 57E at 5 months of age. In none of these cases was there evidence of ocular deformity except for the lenticular opacity. However, the progeny of beagle 57E revealed two outstanding features; namely, that cataract could develop in young puppies (2 to 3 months of age) and that a correlation existed between the size of the eyeball and the severity of lenticular opacity.

The amount of lenticular opacity was in direct relation to the degree of microphthalmia exhibited. This relationship is shown in Figure 1. When compared with the normal eye (Fig. 1, A), cataractous eyes (Fig. 1, B and C) were not only smaller, but the normal curvature of both the cornea and iris was lacking. Measurements of the 46 eyes from puppies used in this study indicated that a correlation between ocular size and lens opacity could be made. These measurements and the degrees of cataract observed by ophthalmoscopic examination are summarized in Table II. The clinical observations were confirmed by histologic study of the lenses. A slight decrease of about 3 per cent from the volume of the normal eyeball size occurred in association with partial cataract, while completely cataractous eyes were 18 per cent smaller than normal.

Microphthalmia and Retinal Folds

Study of cataractous eyes indicated the existence of defective structures of the eyeball rather than the absence of any ocular component. The lesions affected the retina as well as the lens. As shown in Figure

2, the retina (D) was composed of multiple folds or invaginations, and the lens (A) appeared as a heterogeneous mass of material without uniform architectural arrangement.

Figure 2 illustrates the most severe form of microphthalmia and cataract observed in this study. Retinal folds form during embryologic

TABLE II

Severity of Cataract in Relation to Ocular Size

	Longitudinal axis (mm.)	Equatorial axis (mm.)
Normal eye		
Range	16.5–17.5	14.5–16.5
Average	16.8	14.8
Partial cataract		
Range	15.5–16.5	14.5–16.0
Average	16.0	14.6
Complete cataract		
Range	10.0–12.5	10.5–12.5
Average	14.4	11.6

development. The growing retina takes on the internal curvature of the eyeball. Neurones and neuroglial elements proliferate and eventually form the 10 layers of the visual retina, and neuroglial elements, the nonvisual or anterior portion of the retina. In the puppy, retinal development to the stage of permitting vision does not occur until about one week after birth. As shown in Figure 3, the retina in microphthalmic, cataractous eyes continued to proliferate as in the case of the eye of normal size. Cellular proliferation in such instances occurred largely in the outer nuclear layer of the retina, and resulted in invaginations or retinal folds protruding into the vitreous cavity. The other nuclear layer (Fig. 3, A) penetrated or pushed into the bipolar and ganglionic layers (Fig. 3, B and C), and the rod and cone processes formed vesicle-like structures. In eyes in which congenital cataract appeared, embryonic growth of the retina was apparently limited by ocular size, and intrusion into the vitreous was necessary to accommodate the retinal proliferation. Hence, extensive retinal folds were found in all microphthalmic eyes observed in this study. Obviously, such folds could not be capable of photoreception since continuity of the layer of rods and cones did not exist.

Lens Development in Relation to Cataract

According to Mann,[6] congenital cataract should properly be termed developmental cataract, the explanation being that opacities are, in reality, an aberration and not an arrest in development.

Mann[7] described the normal development of the lens, indicating that embryonic development of the human eye began in the 4.6 mm. embryo and progressed by forming a lens pit and lens vesicle; the final stage was the separation of a lens vesicle that showed proliferation of lenticular fibers. The latter was apparent in the 16 mm. human embryo. We have observed similar stages of development in the dog. However, the length of the dog embryo and the stage of gestation should be mentioned in relation to ocular development. The dog embryo at 23 days is 5 mm. long, and at this stage the lens pit is formed. At 27 days (15 mm.) a well-developed lens vesicle containing lenticular fibers is evident. However, only primitive lens fibers, represented by single cells passing from the back to the front portion of the lens, are present. It is not until the fetus is 24 to 25 mm. long (mid-gestation) that secondary lens fibers begin to form. These proliferate from the equator of the lens toward the posterior and central portions, forming the lens suture. According to Cordes,[8] it is during this stage of development that epithelial cells at the periphery of the lens secrete the lens capsule. Cells of secondary lens fibers remain at the equator as the so-called lens bow. This stage of lens development is of concern in this study, since evidence will be presented which indicates that inherited cataract has its beginning as a capsular lens defect.

Histology of Lens Opacity

Histologic examination of cataractous eyes in this study indicated that lens opacity was the fundamental lesion. The size of uveal tract vessels containing erythrocytes revealed an ample blood supply to the eye. The corneoscleral canals were open; if anything, the spaces of Fontana and the canal of Schlemm were slightly enlarged (Fig. 2). The vitreous body and suspensory ligament of the lens appeared normal histologically. However, in the case of complete cataract, the lens appeared as an irregular mass (Fig. 4). The anterior and posterior curvatures of the lens were similar. Normally, the posterior lens curvature is considerably more convex than the anterior. Approximately one fourth of the lens substance was represented by a homogeneous mass containing remnants of abortive lens fibers (Fig. 4, B). This mass lay at the posterior axis of the lens, and anterior to it an incomplete suture line was apparent (Fig. 4, D). The remainder of the lens substance consisted of lenticular fibers in various stages of development. The fibers were extremely variable in size and did not assume a normal pattern. They formed a fibrous mass, having a cumulus appearance rather than a lamellar arrangement. The presence of a suture line and concentrated nuclei at the equator of the lens

suggested that lens opacity was caused by defective secondary lens fibers. With this in mind, the equatorial region of cataractous lenses was studied further.

The normal lens bow, with anterior epithelial cells continuing from the surface to the equatorial region, is illustrated in Figure 6. At the equator, nuclei appear in the substance of the lens within lenticular fibers. As their numbers decrease toward the deeper portion of the lens, the nuclei move anteriorly to form a lens bow. In contrast, the cataractous lens (Fig. 7) showed epithelial cells anterior to the equator where they assumed a very irregular pattern and became dispersed throughout the lens substance instead of forming a lens bow. Some cataractous lenses contained epithelial cells deep within their substance, and occasionally islands of epithelial cells were observed at the posterior axis of the lens. In two cases, the islands were apparent as masses of nucleated lens fibers situated in the hyaloid canal about midway between the lens and optic disc. In one eye a similar mass of tissue protruded from the posterior lens toward the *ora serrata* and encroached upon the posterior ciliary body.

It is well known that the formation of lenticular fibers is dependent upon the presence and continuity of the lens capsule. For instance, rupture of the lens capsule may result in the formation of "Elschnig pearls" after an unsuccessful discission operation.[9] In this study the occurrence of an irregular and poorly developed posterior border of the lens and an ill-defined equatorial zone suggested that a capsular defect might have been responsible for the development of the opacity.

The normal lens capsule stains as a hyaline material with a rich polysaccharide content. The Hotchkiss staining technique for polysaccharide,[2] applied to cataractous eyes, revealed irregular thick and thin areas or loss of continuity of the entire posterior lens capsule. In the case of partial opacity of the lens, a corresponding loss of lens capsule continuity was manifest (Fig. 5). In the latter, lens fibers appeared to be normal in those regions associated with a homogeneous and uniform lens capsule.

Conclusion and Summary

One male (57E) among 1,129 beagles raised under identical conditions[10] showed evidence of developmental cataract. This dog suffered impaired vision and was found to have complete bilateral cataract at 5 months of age. His offspring revealed a large number of cataracts (22 of 25 pups) in various stages of development. Puppies with complete cataractous eyes showed 3 outstanding lesions: microphthalmia, retinal folds, and lens opacity. Puppies with partial cataract showed only slight microphthalmia and retinal folding. This form of cataract

may best be explained as a defect in the development of the secondary lens fibers apparently as the result of incomplete lens capsular continuity. Organs and skulls from all puppies were examined, but ocular defects were the only lesions found.

Similar lenticular lesions may also be found accompanying other types of cataract. Cogan, Donaldson, and Reese[11] described equatorial derangement of cells, ectopic epithelial nuclei, and "balloon cell" formation in radiation cataract. These authors also described a thickening of the posterior capsule as a pathognomonic alteration. Capsular lesions were not found consistently in radiation cataracts, but when evident, appeared as focal fibrillar thickenings. In contrast, congenital cataract consistently revealed posterior capsular deformity. In all instances, the capsular defect in this condition appeared as a homogeneous (hyaline) staining material, lacking in continuity.

The authors wish to express their appreciation to Dr. Samuel J. Kimura, Associate Professor of Ophthalmology, University of California Medical School, San Francisco, for his valuable suggestions; to Miss Eloise Wooten, Senior Laboratory Technician, A.E.C. Project No. 4, for her aid in preparing the specimens; and to Mr. Lorne H. Hardaker, photographer, University of California, Davis, for the excellent photographs.

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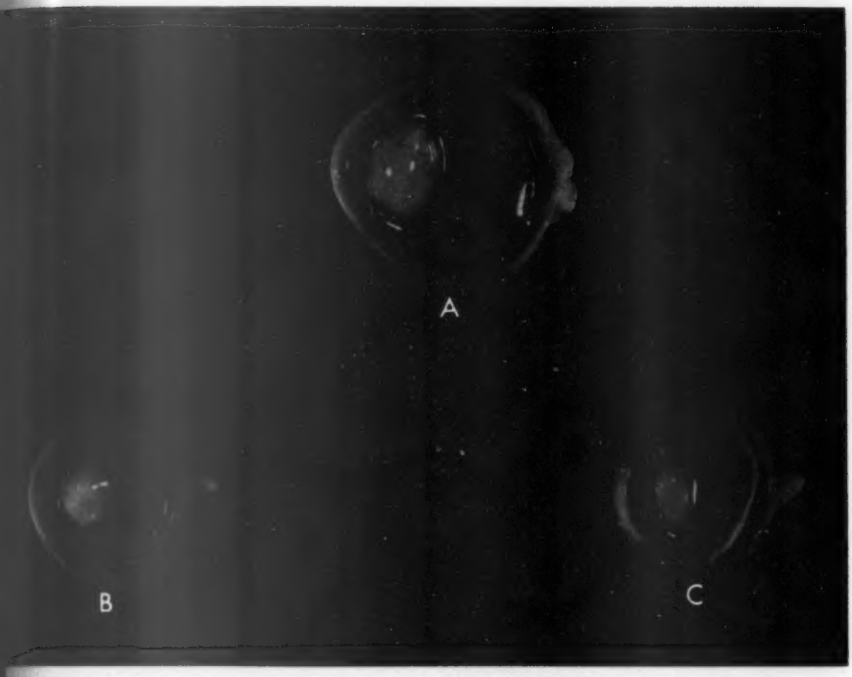
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Normal eye (A) compared with cataractous eyes (B and C). The latter are smaller and lack the normal curvatures of both the cornea and iris. Note the small lens mass within the vitreous cavity (hyaloid canal) in B. Eyes were those of three litter mates (37A, 37B, 37C, 50 days of age). × 2.

FIG. 2. Longitudinal section of cataractous eye. (Dog 29B, 41 days of age.) A, lens; B, iris; C, corneoscleral junction; D, retinal folds. Hematoxylin and eosin stain. × 3.5.

FIG. 3. Retinal fold developing from proliferating outer nuclear cells in an eye with microphthalmia and cataract (puppy 29B). A, nuclei to rods and cones; B, bipolar, and C, ganglionic layers; D, rods and cones; E, choroid; and F, vitreous cavity. Hematoxylin and eosin stain. × 200.



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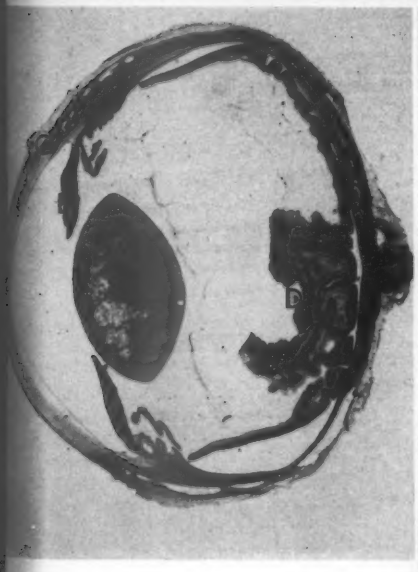

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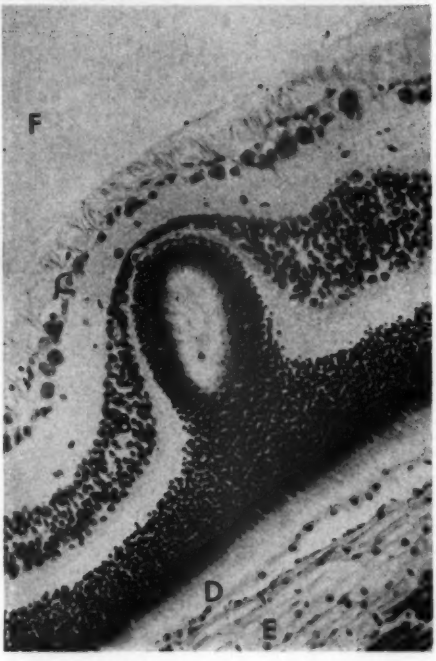

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FIG. 4. Midsagittal section of cataractous lens (puppy 37E, 50 days of age). A, iris; B, posterior lens mass; C, defective lens fibers; D, complete suture line. Hematoxylin and eosin stain. × 10.

FIG. 5. Lens capsule, partial cataract. A, posterior lens capsule becoming thin and losing continuity; B, equator; C, imperfect lens bow; D, relatively normal-appearing lens fibers in contrast to E, defective fibers; and F, homogeneous area. Hotchkiss stain. × 100.

FIG. 6. Lens bow of normal eye (A), (puppy 37A, 50 days of age). × 200.

FIG. 7. Cataractous lens bow (B). Note the abortive attempts to form relatively normal lens fibers at the equator (arrow) with progressive increase in defective fibers ("balloon cells") toward the deeper portion of the lens. Hematoxylin and eosin stain. × 200.

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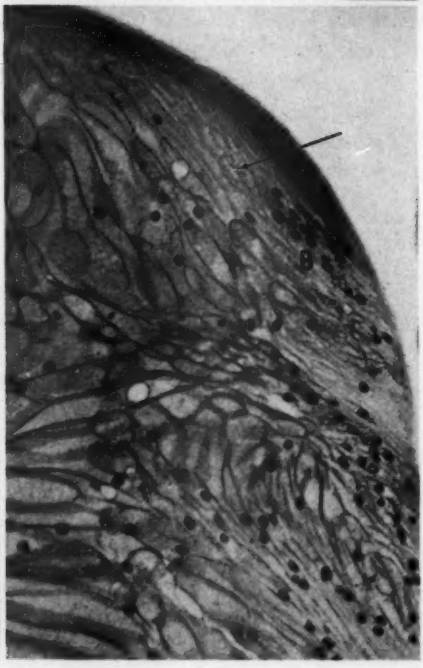

7

IDENTIFICATION OF THE ANATOMIC DEFECT IN PSEUDOXANTHOMA ELASTICUM *

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Pseudoxanthoma elasticum (PXE) is a heritable disorder of connective tissue which commonly affects the skin, fundus oculi, and cardiovascular system. McKusick[1] concluded from his inclusive review that there was probably more than one genotype responsible for PXE, although the most prevalent one is represented by a recessive autosomal gene with some selectivity for the female. The clinical manifestations usually appear in or after the second decade. Most common are the dermal lesions, which appear as thickened, grooved, elevated plaques on the chin, neck, and various flexural folds, notably in the axillary and inguinal areas. Similar alterations may occur in the soft palate as well as in the mucosa of the rectum and vagina.

Ocular involvement is characterized initially by angioid streaking of the fundus and later by varying degrees of choroidal and retinal, particularly macular, degeneration. These are believed to result from ruptures in Bruch's membrane and hemorrhage from abnormal blood vessels.[2]

A frequent observation in these patients is a weakness or absence of pulsations in one or more of the larger peripheral arteries. This is often associated with intermittent claudication in the affected extremities. Roentgenograms have revealed marked calcification of these vessels.

In addition to the peripheral vascular manifestations, hemorrhage may occur in various sites. These include the subarachnoid space, retina, kidney, nasal mucosa, joints, and most importantly, the gastrointestinal tract. The latter may stem from a peptic ulcer, but in many cases the source of hemorrhage is obscure;[3] the common basis is believed to be disease of the gastrointestinal vessels.

The histologic recognition of the cutaneous lesion in PXE is rarely difficult, and the diagnosis may be established readily without recourse to special stains. Band-like collections of hematoxylin staining curlicues and granules within the middle and deeper portions of the corium

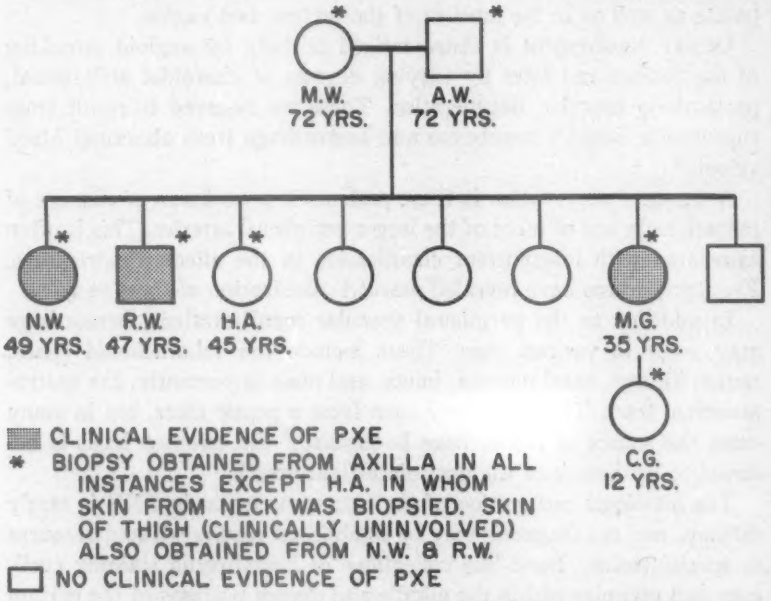
* This investigation was supported in part by a Senior Research Fellowship SF 33C, from the Public Health Service and Grant H 2783 National Heart Institute, Public Health Service.

Presented at the Fifty-fifth Annual Meeting of the American Association of Pathologists and Bacteriologists, Cleveland, Ohio, April 24, 1958.

Received for publication, February 15, 1958.

are characteristic. Tuberculoid or foreign body reactions are occasionally observed in these affected areas. Examination with appropriate staining techniques often reveals the presence of calcium within the lesion (Fig. 1). The fibrillary and granular components of the lesion have a marked affinity for dyes commonly utilized for the demonstration of elastic fibers (Fig. 2). The location of the lesion permits its differentiation from the more common senile elastosis, which appears characteristically in the upper or papillary region of the dermis.

Although most investigators have considered PXE a disorder of elastic tissue, as the name implies, this concept has recently been challenged.[1,4,5] On the basis of electron microscopic studies, Tunbridge and associates[5] concluded that the abnormal fibers were collagenous in nature. McKusick[1] has presented several theoretical objections to the identification of a disorder of elastic tissue as the basic defect in



Text-figure 1. Hereditary transmission of PXE in kindred "W."

PXE and has considered collagen suspect as the tissue primarily affected.

We have had the opportunity to study portions of skin from 7 members of a kindred in whom 3 presented overt dermal as well as ocular and peripheral vascular manifestations of PXE. This report is concerned with the result of histochemical, and fluorescence and electron

microscopic investigation of this tissue. The observations indicate, contrary to recent proposals, that PXE is a primary disorder of elastic tissue rather than of collagen.

Material and Methods

The source of the tissue utilized in this study is depicted in Text-fig. 1. Detailed clinical information concerning this family will be reported elsewhere.[6] A portion of each biopsy specimen was quick frozen in dry ice and sectioned at 10 μ in a cryostat at −20° C. for those techniques requiring fresh frozen sections. Tissue was also fixed in 10 per cent neutral formalin. A portion of the latter was utilized for the preparation of frozen sections; the remainder was dehydrated and embedded in paraffin. All paraffin sections were taken to water in the usual manner, except when otherwise indicated by a particular procedure. Small cubes of tissue from the specimens were also fixed in buffered osmium tetroxide and embedded in butyl methyl methacrylate by the method of Palade.[7] Ultrathin sections were cut, mounted on copper grids and examined first by phase microscopy and then by electron microscopy (Philips EM 100B). In addition, samples of apparently normal skin from the axilla, neck, chest, and abdomen of 6 persons without PXE, undergoing various surgical procedures, were similarly processed, and sections stained simultaneously with those from the persons of the kindred under investigation. Formalin fixed tissue of a dermal lesion from another, unrelated individual with PXE was also available for examination. A variety of histologic and histochemical procedures were performed according to the methods described by Lillie[8] unless otherwise noted (Tables I, II, and III).

Results

The morphologic appearance of the dermal lesion, the various tinctorial reactions, and the observations made by fluorescence and electron microscopy were similar in all examples of PXE studied. These are listed in Tables I, II, and III. A few features which have not been emphasized previously are worthy of mention. It was noted that some fibers within the lesion contained distinct, regularly spaced, optically clear cross bands (Fig. 3). Abundant, coiled reticulum fibers, the so-called *gitterfasern*, were also evident in the lesions in appropriately stained sections. Mast cells were numerous, but other inflammatory cells and fibrocytes were sparse. The acid mucopolysaccharide identified within the lesion appeared as small "pools" but also coated fibrillary and granular elements, and, for the most part, assumed their configuration (Fig. 4). The vessels of the corium and subcutaneous

TABLE I

Tinctorial Characteristics of Pseudoxanthoma Elasticum (PXE), Normal Elastic Tissue and Collagen

Procedure	PXE			Normal	
	Lesion	Elastica	Collagen	Elastica	Collagen
Lipid stains					
Oil red O	–	–	–	–	–
Sudan black B	–	–	–	–	–
Schultz	–	–	–	–	–
Fischler	–	–	–	–	–
Paracetic acid Schiff	–	–	–	–	–
Baker's acid hematein	–	–	–	–	–
Polarized light	–	–	A	–	A
Polysaccharide stains					
Periodic acid-Schiff	–/+*	–	±	–	±
Thionine	M	O	–	O	–
Streptococcal hyaluronidase	–	O	–	O	–
Testicular hyaluronidase	–	O	–	O	–
Ribonuclease	M	O	–	O	–
Elastase	M	–	–	–	–
Acetylation and deamination	M	O	O(±)	O	O(±)
Rinehart-Abul-Haj	±	±	±	±	±
Streptococcal hyaluronidase	±	±	±	±	±
Testicular hyaluronidase	±	±	±	±	±
Mineral stains					
Von Kössa	+	–	–	–	–
Elastase	+	–	–	–	–
0.1 N hydrochloric acid	–	–	–	–	–
Perls's	–	–	–	–	–
Ferric ferricyanide	–	–	–	–	–
Enzymes					
Alkaline phosphatase	–	–	–	–	–
Succinic dehydrogenase?	–	–	–	–	–
Esterase	–	–	–	–	–
Protein stains					
Ninhydrin	–	–	+	–	+
Ninhydrin-Schiff	–	–	+	–	+
Danielli	±/–	±/–	+	±/–	+
Phosphotungstic acid hematoxylin	Blue	Blue	Orange	Blue	Orange
Masson	Yellow	Pink	Green	Pink	Green
Other stains		(reticulum?)		(reticulum?)	
Harris alum hematoxylin	+	–	–	–	–
0.1 N hydrochloric acid	–	–	–	–	–
Ribonuclease	+	–	–	–	–
Hematoxylin without alum	±	–	–	–	–

Key: + = Positive; – = Negative; A = Anisotropic; M = Metachromatic; O = Orthochromatic. * PAS-positive in "pools" only.

TABLE II

Effect of Enzymes and Blocking Reactions on Elastic Tissue Staining

Procedure	Verhoeff-van Gieson			Aldehyde fuchsin			Orcein		
	PXE	Elastica	Collagen	PXE	Elastica	Collagen	PXE	Elastica	Collagen
Untreated	Black (++++)	Black (++++)	Orange Red (++++)	Purple (++++)	Purple (++++)	Purple (−)	Brown (++++)	Brown (++++)	Brown (−)
Streptococcal hyaluronidase	++++	++++	++++	++++	++++	−	++++	++++	−
Testicular hyaluronidase	++++	++++	++++	++++	++++	−	++++	++++	−
Elastase – 2, 4, 6 hrs.	±	−	++++	±	−	−	±	−	−
Preceded by 0.1 N HCl	−	−	++++	−	−	−	−	−	−
Trypsin (Difco) – 2, 4, 6 hrs.	±	−	++++	±	−	−	±	−	−
Trypsin (Worthington)	++++	++++	++++	++++	++++	−	++++	++++	−
Pepsin									
1, 2 hrs.	−	−	++++	−	−	−	−	−	−
4 hrs.	−	−	+	−	−	−	−	−	−
Deamination – 6 hrs. 60°C. and 24 hrs. 25°C.	++++	++++	Black +	++++	++++	++	++++	++++	+++
Acetylation – 6 hrs. 60°C. and 24 hrs. 25°C.	++++	++++	Black ++	++++	++++	−	++++	++++	+++
Methylation									
6 hrs.	−	−	++++	±	±	−	++++	++++	−
24 hrs.	−	−	++	−	−	−	++++	++++	−

tissue appeared unaltered. Examination of representative portions of the lesions by electron microscopy revealed fibers lacking the characteristic periodicity of collagen (640 Å) (Fig. 5).

No evidence of PXE was apparent in sections of skin prepared from members of this kindred who had no clinical manifestations of the

TABLE III

Effect of Various Treatments on Autofluorescence[19]

	PXE	Normal elastica	Collagen
Untreated	++++	++++	±/−
Acetylation	++++	++++	±/−
Deamination	++++	++++	±/−
Methylation	+++	++++	±/−
Elastase	−	−	±/−
Pepsin	−	−	±/−
Hyaluronidase	++++	++++	±/−
Pyridine 24 and 48 hrs. at 60°C.	+++	++++	±/−
Chloroform-methanol 24 and 48 hrs. at 60°C.	+++	++++	±/−
0.1 N hydrochloric acid	++++	++++	±/−

disorder. However, in the case of patient N. W., a classical lesion was noted deep in the corium and in the adjacent subcutaneous tissue of the thigh, a site exhibiting no clinical evidence of the disorder. No morphologic alteration was observed in the grossly unaffected skin of the other patient (R. W.) from whom a biopsy specimen was obtained. There were no apparent differences in the tinctorial reactions of the elastic fibers of skin obtained from various sites from persons without familial or clinical evidence of PXE. The reactions noted in the elastic fibers in the latter group were found to be similar to those observed in unaltered elastic fibers in sections of skin containing the classical lesion of PXE.

DISCUSSION

The results of this study are considered to provide evidence that the fundamental defect in the dermis of pseudoxanthoma elasticum resides within elastic fibers, and not in the collagenous connective tissue as has been suggested recently. The relative paucity in the skin of elastic as compared to collagen fibers, and the fact that the highest concentration of the former lies immediately beneath the epidermis do not constitute valid criticisms of the contention that PXE is primarily a disorder of elastic tissue. Histologic examination of appropriately stained sections of normal human skin from those areas frequently affected in PXE, such as the axilla and neck, reveals a sufficient num-

ber of elastic fibers within the deeper portions of the corium to account for the alteration observed at this site. It seems apparent that disruption and fragmentation of these fibers could well account for the magnitude of the lesion encountered in PXE without postulating that it be derived from a quantitative increase of such structures. The detection of cross bands in some of the distorted fibers in formalin-fixed sections of the lesion is strikingly reminiscent of the elastic fibers in formalin-fixed sections of the lesion of normal human skin and the *ligamentum nuchae* during the process of digestion with elastase.[11,12] This pattern was also evident in decalcified sections. If the lesion were derived from collagen, one might cogently inquire as to the fate of the elastic fibers since normal elastic components are sparser in the skin harboring the lesion of PXE than in control sections from similar sites.

The weakly positive or negative reactions to staining for amino acids and protein observed in normal elastic fibers and those comprising the lesion of PXE do not preclude the presence of these moieties. However, this observation may be interpreted as indicating that these reactive groups are more widely spaced in elastica than in collagen, as suggested by Pearse.[13] This represents another tinctorial difference between normal elastic fibrils and those in PXE and the staining of collagen; the latter is more strongly reactive.

The significance of the affinity for elastic tissue dyes of the fibrillary and granular components of the lesion in PXE in relating this disorder to an alteration of elastic fibers has recently been questioned. Gillman, Penn, Bronks, and Roux[14] concluded that the "basophilic" fibers occurring in the dermis following irradiation and in degenerating arteries, considered heretofore to represent an alteration of elastica, were formed rather from degenerated collagen. In addition to the affinity for the usual elastic tissue dyes of such fibers, they noted that they were also colored by some dyes which do not stain normal elastic fibers. Moreover, it was observed that "elastotically degenerated fibers" were not cyanophilic in sections stained by the phosphotungstic acid hematoxylin technique. However, such considerations do not eliminate the possibility that altered elastic fibers may exhibit atypical staining reactions, as emphasized by Hass.[15] We have constantly observed the fibers of the lesion of PXE to be cyanophilic when stained by the phosphotungstic acid hematoxylin method. Further, it is well recognized that this technique may exhibit blue as well as orange staining components in some fibers which are unequivocally of elastic nature. Whether this disparity represents a fundamental difference between the lesion of PXE and those conditions studied by Gillman and associates[14] or results from variations in the technique utilized cannot be

stated. It is apparent from this study, as well as from that of Fullmer and Lillie,[16] that collagen may exhibit an affinity for some elastic tissue dyes following acetylation or deamination, procedures which block basic polar groups. It appears significant, however, that collagen failed to exhibit any of the other characteristics observed in the abnormal fibers of PXE or in normal elastica following chemical treatment of this nature. Moreover, prolonged periods of acetylation failed to induce staining of collagen by aldehyde fuchsin. The fibrils in PXE differed in no way from normal elastica in staining affinity with the elastic tissue dyes utilized. It appears unlikely, therefore, that a comparable alteration of collagen constitutes the fundamental defect in PXE. The data suggest rather that the affinity of the fibers for elastic tissue dyes is indeed due to their elastic nature. This contention is further supported by the observation that both the fibers in PXE and those in the normal elastica lose their affinity for elastic tissue dyes following methylation.

More direct evidence identifying elastic tissue as the site of the defect was provided by fluorescence and electron microscopy. The autofluorescence in the lesion of PXE was identical to that observed in normal elastic fibers (Fig. 6); it disappeared following the application of elastase and pepsin, substances which remove these structures from tissue sections. In contrast, collagen was devoid of autofluorescence or revealed it only faintly in fibers adjacent to elastica. A similar result was noted in sections stained with the fluorescent dye, acridine orange. The nature of the fluorescence of elastic fibers has not been established although it has been suggested that it may be related to a lipid content.[18] It is of interest in this regard that we were unable to demonstrate lipid in either the lesion of PXE or in normal elastic fibers; this is similar to the results of others.[18,17] Prolonged efforts at extraction with pyridine or chloroform methanol failed to inhibit autofluorescence. It would appear, therefore, that if the autofluorescence were dependent upon the lipid content of elastic tissue, this moiety must be firmly bound to protein. It should be noted that lipid has been demonstrated histologically with osmic acid in apparently normal elastica, and also in very small amounts by chemical analysis.[13]

The absence of periodicity in the fibers comprising the lesion of PXE and the resemblance of the fibers to unaltered elastica in sections examined by electron microscopy are observations which differ from those of Tunbridge and associates.[5] They observed collagen fibers which were apparently normal within the lesion in sections of skin from 3 patients with PXE. This apparent divergence may be due to the fact that very limited fields can be examined by electron microscopy.

Since both elastic and collagen fibers are present within the lesion, proper identification of the fibrillary components is paramount. The preliminary utilization of phase microscopy for locating suitable landmarks, as performed in this study, adds further reliability to our observations that the altered fibers are elastic in nature.

The metachromatic material observed about the fragmented fibers in PXE may be characterized as nonsulfated acid mucopolysaccharide by virtue of its lability to both streptococcal and testicular hyaluronidase. The presence of acid mucopolysaccharide in foci of damaged elastic tissue is well recognized, and Wislocki, Bunting and Dempsey[18] could find no exceptions to this association. This material may be the product of fibroblasts, a popular concept, although it should be noted that these cells are sparse in PXE. Hall, Reed and Tunbridge[19] noted the liberation of both polysaccharide and protein during the course of degradation of elastin *in vitro*. This suggests another source for this substance. Although there are a moderate number of mast cells present within the lesion, there is no evidence to indicate that the abnormal material is derived from such cells. The tinctorial reactions of mast cell granules are notably different from those of the acid mucopolysaccharide; there is a strong periodic acid-Schiff reaction as well as resistance to digestion by both testicular and streptococcal hyaluronidase.

The presence of acid mucopolysaccharide and a reticulum network in the lesion might suggest that the disorder represents an alteration in the production of collagen, since such substances are found in the substrate phase of collagen formation. It appears significant, however, that neither alkaline phosphatase nor succinic dehydrogenase were present at these sites. The relationship of these enzymes to collagen production has been well described by Bunting and White[20] and by Argyris.[21] Their presence in the usual sites in the skin indicates that there is no primary defect in these enzymes.

The association of calcium salts with altered elastic fibers is well recognized.[16] The presence of such salts might be responsible in part for the basophilia of the fibrillary components of the lesion when stained with hematoxylin and eosin. Hematoxylin prepared without alum also colors the lesion although not as intensely as alum hematoxylin. The staining effect of the latter was abolished in sections treated with dilute acid sufficient to remove the calcium although the affinity of the fibers for elastic tissue dyes was unaffected. Digestion by ribonuclease, hyaluronidase, or elastase had no influence on staining with alum hematoxylin. These qualities suggest that the hematoxylinophilia of the fibers is in large part due to calcium laking.

The dissolution of the fibrillary elements in PXE by elastase (Fig. 7) was in keeping with the findings of Findlay.[22] Also in agreement was the slightly greater resistance to this enzyme of the abnormal than the normal elastic fibers. This had suggested to Findlay that the altered elastic fiber in PXE was "tougher." It is also possible, however, that this may reflect a difficulty of penetration of the enzyme into the fiber because of its coating with calcium and mucopolysaccharide. Indeed, prior removal of calcium from the sections with weak acid in advance of application of elastase permitted prompt and complete elastolysis. Elastase had no apparent effect on the structural or tinctorial characteristics of collagen. The action of elastase has been attributed to its mucase content. It is of interest, however, that enzymatic treatment sufficient to remove practically all of the fibers in the lesion of PXE and in normal elastica had no effect upon the mucopolysaccharide-dependent metachromasia of the former. Complete removal of the elastic fibers was promptly induced by peptic digestion (Fig. 8); its elastolytic effect in the concentrations employed was about 4 times greater than its effect on collagen, and about twice as great as the elastolytic activity of elastase itself. The elastolytic activity of pepsin has not been generally appreciated, at least within the periods of incubation utilized in this study. This effect has also been observed *in vitro* and will be discussed in greater detail elsewhere.[23] The removal of elastica from tissue sections by one of the trypsin preparations utilized, but not by the other, a more highly crystallized form, suggests that the former had an appreciable elastase content.

It appears, thus, that the fibers affected in pseudoxanthoma elasticum possess many of the physical and histochemical characteristics which are ascribed to elastic tissue. A number of these are notably similar to the tinctorial features of degenerated, "senile" vascular elastica.[11] There is little or no evidence to suggest a relationship to collagen, or that the lesion results from an abnormality in collagen formation. This does not preclude the existence of some alteration of collagen, since the abnormal elastica is contiguous with collagen fibers. On the other hand, if changes were indeed present, they were not detected by the methods utilized in this study. The term pseudoxanthoma elasticum appears, therefore, to appropriately describe the dermal lesion of this disorder.

This study has failed to yield specific information concerning the pathogenesis of PXE. However, the presence of characteristic elastic tissue alterations in the normal-appearing skin of the thigh of one patient with PXE suggests that exogenous factors account for the

overt manifestations of the lesion at sites of flexural stress. The lack of lesions in the skin of unaffected members of this kindred substantiates the recessive nature of this heritable disorder.

Summary

The results of histochemical procedures and of fluorescence and electron microscopy performed on sections of the skin from 4 patients with pseudoxanthoma elasticum, 3 from the same kindred, indicate that this disorder represents a primary defect in elastic tissue. The similarity of the fibrillary and granular components of the lesion to those in normal elastica was evident by their affinity for elastic tissue dyes. Both exhibited negative or weakly positive protein reactions, identical susceptiblity to elastase and peptic digestion, and like responses to various blocking reactions. Further similarity was manifested by autofluorescence and lack of periodicity in the fibrils. The significance of these as well as other observations which serve to relate the lesion to an alteration of elastic rather than collagen fibers is discussed.

Lesions were not encountered in the skin of members of this kindred who had no overt evidence of this disease. However, biopsy of an apparently unaffected segment of skin in a patient with dermal involvement at other sites revealed the classical lesion of elastica. Although PXE represents an abiatrophy, it appears that exogenous factors play a role in the pathogenesis of the lesions since they are characteristically located at sites of flexural stress.

The authors are grateful for the technical assistance of Messrs. John Colowit, L. Ferra and James Apicella.

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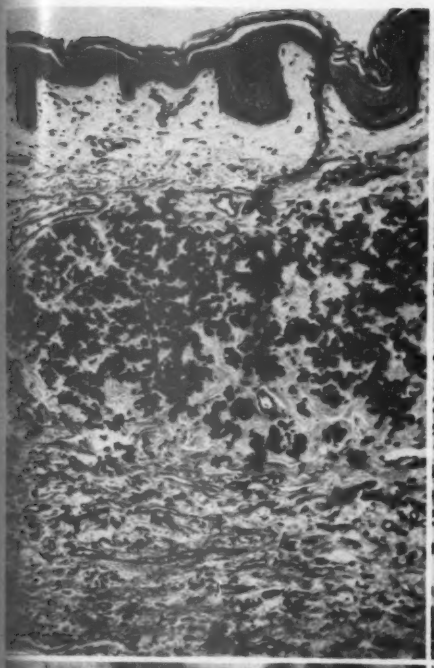
LEGENDS FOR FIGURES

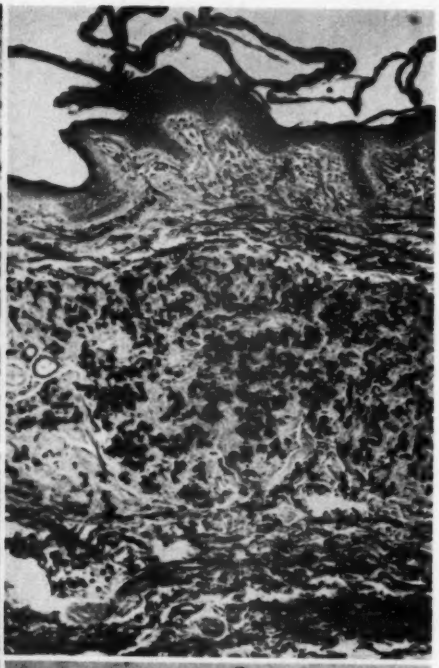
FIG. 1. Calcium (appearing black) coating fibrillary and granular components of lesion of PXE. Von Kóssa stain. × 75.

FIG. 2. Classical dermal lesion of PXE. Verhoeff-van Gieson stain. × 75.

FIG. 3. Cross banding of fibers of lesion of PXE. Orcein, untreated. × 1,200.

FIG. 4. Metachromasia of fibrillary and granular components of lesion of PXE (appearing black). Thionine. × 175.





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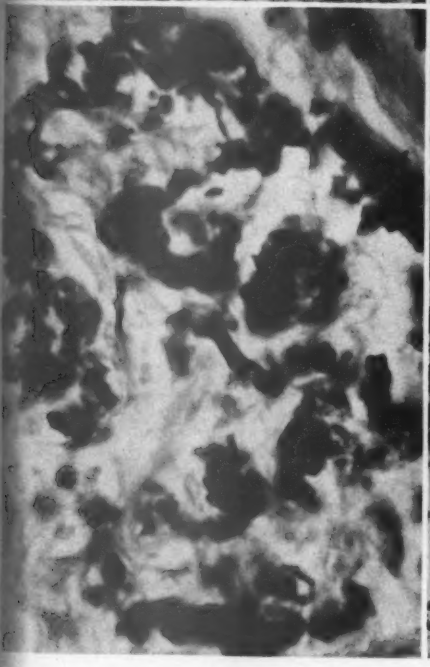

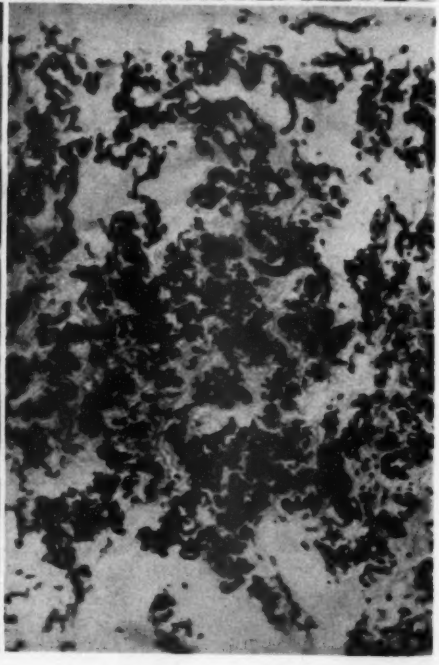

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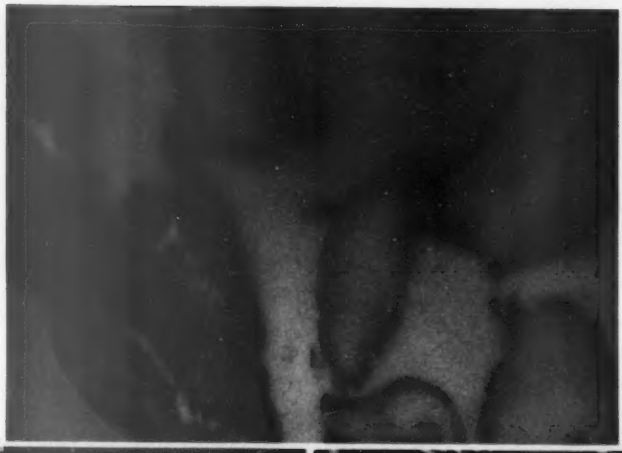
FIG. 5. Electron microphotograph of lesion of PXE, revealing amorphous elastic fibers as well as collagen at left. × 8,000.

FIG. 6. Autofluorescence of components of lesion of PXE (Fig. 6a) is similar to that noted in normal elastic fibers (Fig. 6b). Collagen is negative or only weakly autofluorescent. × 185.

FIG. 7. Section of lesion of PXE treated with elastase prior to staining with Verhoeff-van Gieson stain. Only a few fibrillary remnants remain. × 165.

FIG. 8. Section of lesion of PXE treated with pepsin prior to staining with Verhoeff-van Gieson stain. Fibrillary and granular elements are no longer evident as in Figure 2. × 75.



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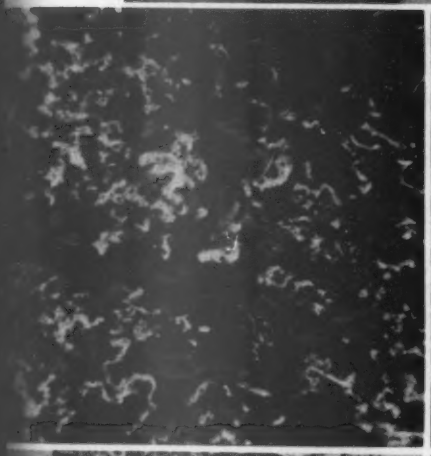

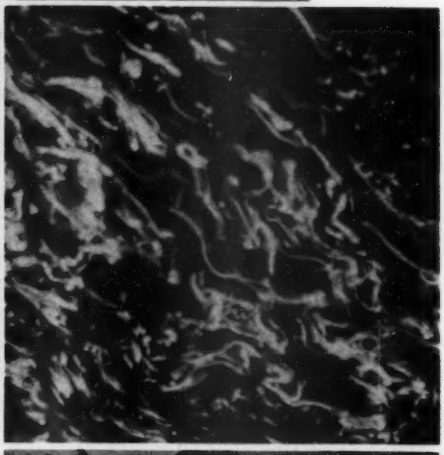

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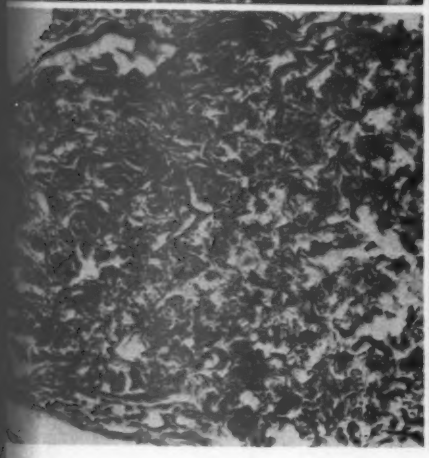

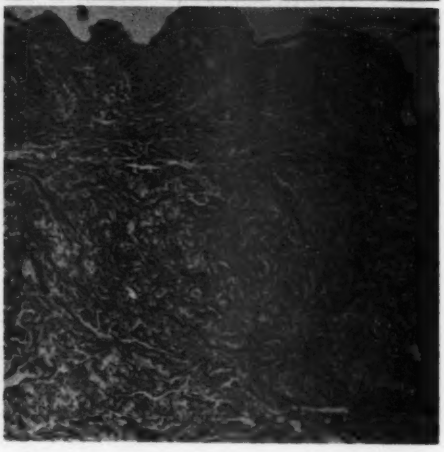

8

THE MUCOPOLYSACCHARIDES IN THE NORMAL AND DISEASED BREAST

Their Distribution and Significance *

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The stroma of the breast has attracted a great deal of attention in the recent past, and there have been numerous reports concerning its morphologic and functional patterns. The existence of a specialized stroma within lobules and around ducts, which differs from the rest of the breast stroma, has long been recognized[1,2] and has led to the identification of an intralobular (and periductal) and an interlobular stroma. The former is responsive to hormonal stimulation and plays a functional role in the cyclic activities of the gland. The morphologic alterations characterizing these physiologic phenomena have been amply described,[3-12] and the variations observed in the intralobular and periductal stroma during the development of dysplastic and neoplastic lesions have been recorded.[4,5,9,10,13]

Recently metachromasia has been noted in intralobular and periductal stroma[14] and has been found susceptible to partial removal by testicular hyaluronidase. This has been related to the presence of acid mucopolysaccharides.[15,19]

In the present study, the distribution and interrelations of the acid and neutral mucopolysaccharides in the breast have been investigated by means of histochemical techniques. The modifications of these substances in the normal functional state of the gland and in its pathologic variations have been observed. At the same time an attempt has been made to correlate the histochemical characteristics with known morphologic features.

Material and Methods

The material consisted of surgical specimens received in the Department of Pathology of the New York Medical College. The tissue was obtained from patients in the Flower and Fifth Avenue Hospitals who had either undergone excision biopsy of breast lesions or radical mastectomy. In all of the female patients, ranging in age from 12 to 73 years, an accurate menstrual or menopausal history was available. Included were 31 specimens of mammary dysplasia (fibrocystic

* Received for publication, February 10, 1958.

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disease and sclerosing adenosis), in 17 of which normal breast tissue was also available for study. In addition there were 10 examples of fibroadenoma (5 including normal breast tissue) and 23 instances of carcinoma (1 noninfiltrating lobular carcinoma; 10 infiltrating duct cell carcinomas with foci of noninfiltrating neoplasm; and 12 infiltrating duct cell carcinomas). In 20 of these, non-neoplastic breast tissue was available. There were also 10 specimens of gynecomastia. Three examples of normal female breast and 1 male breast were obtained during plastic surgical procedures (Table I). In each instance, a varying number of blocks were used for the investigation. Also examined, but not taken into account in the tabulation of results, were numerous breasts of patients from whom an adequate menstrual history was not available.

TABLE I
Distribution of Cases by Age *

Diagnosis	Age in years			Total
	12–30	31–50	51–73	
Normal breast tissue	8†	28	9	45
Mammary dysplasia	5	24	2	31
Fibroadenoma	6	4		10
Fibroadenomatous hyperplasia	5			5
Carcinoma		11	12	23
Gynecomastia	5	1	4	10

* Only the cases with acceptable menstrual or menopausal history are tabulated.
† One case is of male breast.

Tissue was fixed in 10 per cent neutral formalin and embedded in paraffin. The sections were cut serially at 4 μ and attached to slides in an alternate order to allow the closest comparison between sections of each block stained by various methods. The sections were affixed to the slides with distilled water to avoid the use of glycerin-albumin.

Sections from each block were stained with hematoxylin and eosin, and by the Ritter-Oleson (RO) method.[20] The latter combined Hale's technique (HS)[21] for the staining of acid mucopolysaccharides and the periodic acid-Schiff stain (alcoholic solution of periodic acid) for the demonstration of neutral mucopolysaccharides. In addition, many sections were stained by the PAS method alone (aqueous solution of periodic acid) and with toluidine blue.

The carbohydrate nature of the PAS positive substances was tested by the acetylation technique proposed by McManus and Cason.[22] The identification of acid mucopolysaccharides in HS positive material, as well as in the structures stained metachromatically with toluidine blue

(TB), was checked by incubation in various concentrations of hyaluronidase prior to the staining. The enzymes used were testicular hyaluronidase (1,000 turbidity reduction units [TRU] per mg.) and pneumococcal hyaluronidase (450 TRU per mg.) which were kindly furnished by Dr. K. Meyer of Columbia University.

Results

Normal Female Mammary Gland

In a female mammary gland, regardless of the stage of evolution, the ducts and acini are enveloped in a well outlined network of fine fibers constituting the basement membrane. In sections stained by the PAS and RO methods, these fibers stood out because of their intense red-purple color.

The fibers appeared to be arranged along the ducts in longitudinal and circular manner, better seen in ducts cut tangentially (Fig. 1). They were closely interlaced to form a tight network in which it was impossible to decide whether the longitudinal or circular fibers were external, one to the other, or whether they were interlaced in a single layer. The fibers were in close contact with the myo-epithelial cells, and fibrils were interposed between them, approaching, but never reaching, the lining epithelium. No orderly pattern was observed in the basement membranes of acini. At this level, the membranes were formed by fibers running in various directions.

Surrounding the ducts and acini, external to the basement membrane, the stroma, in general, stained in positive fashion with RO and HS and also proved to be metachromatic. A few mast cells were visualized more clearly with toluidine blue than with the RO stain.

The interlobular stroma was predominantly PAS positive. Stromal elements stained blue with the RO method, and there was less metachromasia than in the periductal stroma.

Of interest were the changes observed in the intralobular stroma during the menstrual cycle. These alterations have been described by Foote and Stewart.[12] They were evident in our sections stained with hematoxylin and eosin and were correlated chronologically with the menstrual histories. It was noted that the interlobular stroma did not change its reaction to any of the stains to a significant extent, but showed large amounts of PAS positive material throughout the cycle. The intralobular stroma, on the contrary, showed characteristic cyclic alterations. During the "resting" (intermenstrual) phase, the intralobular stroma appeared to be in part PAS and in part HS positive, the former predominating somewhat over the latter. As the "evolutional" (premenstrual) phase began (Fig. 2), increases in HS posi-

tivity and metachomasia were noted. This increase continued gradually almost to the time of menstruation, while the PAS positive material disappeared within the first few days of the phase. The rise in the amount of HS positive material was concomitant with an increase in looseness of the stroma and in the number of fibroblasts. Mast cells were also seen, but their numbers did not appear to increase significantly. During the last few days of the "evolutional" phase, PAS positive secretory granules appeared within the acinar cells and there was an accumulation of secretory material with similar staining quality in the lumens (Fig. 3). The PAS staining resisted diastase digestion.

In the "involutional" (postmenstrual) phase, the reverse of these modifications took place, with gradual restoration to the pattern of the resting phase. No impressive changes were noted in the basement membranes which remained PAS positive during all phases of the cycle.

In summary, the HS positive and metachromatic substances of the intralobular stroma increased in amount progressively from approximately the 12th or 15th day of the cycle to the time of menstruation, and then decreased gradually to reach the low amount characteristic of the intermenstrual stage at approximately the 10th or 12th day after menstruation. On the contrary, the PAS staining of the stroma gradually disappeared by about the 16th or 18th day after menstruation, to reappear at the time when the HS positive substances reached their lowest amount.

In a case of pregnancy, at the fifth month, the stroma exhibited a pattern analogous to that in the menstrual stage. In breasts of patients immediately after term delivery, little stroma was present between the glandular structures. Therefore, it was difficult to evaluate the histochemical pattern accurately, but it appeared that this stroma was metachromatic and in part PAS and in part HS positive.

During menopause (Fig. 4), the metachromatic and HS positive material tended to decrease in amount and reached a stage in which there was no difference in staining properties between the intra- and the interlobular stroma. In this age group, only on very rare occasions were there evidences of HS staining and metachromasia in the periductal stroma.

Mammary Dysplasia

Under this heading are included a heterogeneous group of nonneoplastic lesions, fundamentally similar to each other in that they may be considered morphologic manifestations of an "abnormal interplay of parenchyma and stroma," expressed by "failure of reciprocal proliferation and involution."[28] They represent a possible expression

of longstanding hormonal imbalance (mainly ovarian) often associated with other constitutional factors.[24,25]

In the cases studied, the interlobular stroma showed no histochemical difference from the staining observed in normal breasts and was essentially PAS positive. The most frequent change, common to all the lesions, was noted in the intralobular and periductal stroma, which no longer showed cyclic alterations. This was particularly evident when comparing lesions with nearby normal breast tissue. In lesions of short duration, HS staining and metachromasia were still demonstrable in the intralobular stroma but appeared unrelated to the phases of the menstrual cycle. In advanced cases, these staining qualities were progressively reduced until only PAS positive material remained. At this stage there was no significant staining distinction between interlobular and intralobular stroma. Not all the cases, however, exhibited so orderly a progression of events. In some long standing lesions, metachromasia and HS positivity remained around the ducts. It is emphasized, however, that in these cases the presence and the intensity of the HS reaction and metachromasia did not correspond to the phase of the menstrual cycle on the day of operation. On the other hand, in contiguous normal breast tissue the cyclic characteristics were manifest.

The basement membranes of abnormal ducts and acini, and of cystic structures as well, were well preserved in all forms of mammary dysplasia (Fig. 6). Some changes, such as thinning, swelling, and poor definition, were noted, but these did not seem related to any particular morphologic expression of the disease. Rather, it seemed that the basement membranes were the seat of minor abnormalities when the myo-epithelial elements of affected structures were irregularly oriented or had disappeared. Sections of sclerosing adenosis stained with the PAS and the RO methods were of particular interest. In the florid and sclerosing stages, and with no relation to the severity or the extent of the lesions, the basement membranes were always remarkably well-stained, although disoriented by epithelial proliferation or fibrosis (Fig. 7). This was particularly striking when sclerosing adenosis was present in a breast containing carcinoma which was found to be devoid of basement membranes.

Secretory activity was well demonstrated in cysts, especially when these were lined by metaplastic apocrine epithelium (Fig. 5). In this type of epithelium, diastase resistant granules stained with PAS were practically always evident, particularly in the bulging portion of the cell facing the lumen. Frequently, the cyst content was also PAS positive and occasionally stained with HS as well (Fig. 6).

Fibroadenoma and Fibroadenomatous Hyperplasia

In the relatively small number of fibroadenomas examined, staining characteristics were quite constant. In pre-menopausal patients, the stroma surrounding the ducts regularly showed a marked HS staining, intense metachromasia, and the absence of PAS positive structures. The remainder of the stroma, corresponding to that in the interlobular region, contained equal amounts of PAS and HS staining material. In two cases the stroma was only HS positive, as in the periductal region. In one specimen, from the only patient in menopause, the stroma in both locations showed identical reactions, with equally distributed positivity to the PAS and HS stains. In this case the periductal tissue exhibited a reaction similar to that of interlobular stroma. It is of interest that in the stroma of the adjacent normal tissue in this breast, which was the seat of menopausal involutional changes, there was identical reaction to the RO stain. In every case, the basement membranes of ducts appeared well defined. No significant abnormalities were noted in the epithelial elements.

In 5 instances, the breasts of young patients were the seat of "fibroadenomatoid hyperplasia." In 3 of these cases there was also an independent fibroadenoma. The lesion was characterized by focal proliferations of the stroma surrounding essentially normal ductules. This mimicked the pattern of fibroadenoma, but faded at the periphery into the surrounding normal stroma instead of being sharply demarcated. The proliferated stroma in all 5 instances exhibited marked HS positivity and also considerable metachromasia.

Carcinoma

Carcinoma of the breast, no matter what the histologic type or age of the patient, usually showed marked HS positivity and metachromasia in its stroma. Only a very small amount of PAS positive material was noted. In one specimen of infiltrating duct cell carcinoma, the stroma exhibited zones of marked HS positivity alternating with areas of PAS staining. The latter, in sections stained with hematoxylin and eosin, were considered to be the seat of desmoplasia.

In predominantly intraductal neoplasms, intense HS positivity and metachromasia appeared in a band of stroma surrounding the neoplastic ducts, while the intervening connective tissue was in part HS and in part PAS positive. Similarly, in lobular carcinoma *in situ*, the intense HS positivity and metachromasia were restricted to the intralobular stroma.

In many cases the metachromatic stroma contained a considerable number of mast cells, particularly about foci of noninfiltrating duct

cell carcinoma. The number of these cells, however, was rather inconstant; they were numerous in some cases and sparse in others. Even in the same section their number varied from one field to another. The staining of non-neoplastic tissue in these breasts was identical with that of normal breasts in patients of comparable ages.

Groups of infiltrating cells in any type of carcinoma were never surrounded by basement membranes. On the other hand, basement membranes were evident in noninvasive carcinoma, of both duct cell and lobular nature (Figs. 8 and 9). In some lesions, the membrane was well stained and PAS positive; in others, it appeared thinned out and partially or totally HS positive. In the latter instance the basement membrane was occasionally discontinuous, and neoplastic epithelium was in process of early stromal invasion.

Intracytoplasmic staining was encountered in only a few cancer cells, particularly in the medullary type of carcinoma.

Gynecomastia

The age range of the 10 cases of gynecomastia was 12 to 62 years. In 8 specimens there were marked metachromasia and HS staining of the periductal stroma, identical to that encountered in the female during the premenstrual stage. In one of the 8, there was also a minimal amount of PAS positive stroma. In the remaining two patients, aged 20 and 24 years, the periductal stroma showed a predominance of PAS staining; metachromasia and reaction to the RO stain were scanty.

The basement membrane of the ducts was always clearly defined and strongly PAS positive (Fig. 10). No abnormalities were noted in the epithelial cells.

In all the cases, the blue color of the stroma with the RO stain, as well as the metachromasia, failed to appear when the tissue was pretreated by incubation with testicular hyaluronidase. Pneumococcal hyaluronidase, used in identical manner, prevented the staining only in part. Acetylation of the sections, according to the method of McManus and Cason,[22] prevented the PAS staining of stroma, basement membranes and secretory precipitate. Staining was restored when acetylation was followed by treatment with alkali.

Discussion

In the evaluation of results, we have had to take into consideration limitations of the techniques utilized. Fixation in formalin permits only a qualitative evaluation and not an accurate quantitative appraisal. Moreover, the RO method for staining acid mucopolysaccharides is admittedly of limited specificity.[26,27] It is felt, however,

that the use of supplementary techniques reduced the limitations to a minimum. The structures reacting with the Hale stain were also metachromatic with toluidine blue and were affected by hyaluronidase. It seemed reasonable to conclude, therefore, that these tissues contained acid mucopolysaccharides. The existence of acid mucopolysaccharides in breast stroma has been suggested by several investigators[15-19] on the basis of metachromasia which is totally or partially eliminated by hyaluronidase. We noted, however, that breast stroma which lost its metachromasia and HS staining when treated with testicular hyaluronidase lost these staining qualities only partially when pneumococcal hyaluronidase was used in similar fashion. Since chondroitin sulfate B is not affected by either testicular or pneumococcal hyaluronidase,[28] its existence is unlikely in this location. On the other hand, hyaluronate and chondroitin are susceptible to both hyaluronidases.[29] Chondroitin, originally isolated by Davidson and Meyer from bovine cornea,[20] is difficult to detect by histochemical methods. However, since it is considered a possible precursor of chondroitin sulfate, it seems reasonable to presume its existence in breast stroma.

Acid mucopolysaccharides resistant to pneumococcal hyaluronidase were demonstrated in our material, but chondroitin sulfate B was not. It appeared, therefore, that acid mucopolysaccharides labile to testicular, but resistant to bacterial, hyaluronidase, such as chondroitin sulfate A and chondroitin sulfate C,[30] were undoubtedly present. It was difficult, however, to establish the presence of both or only one of these. According to Meyer, Davidson, Linker and Hoffman,[31] the two chondroitin sulfates are closely related. They exhibit only a few differences in their chemical or physical features, but their distribution in the tissues varies to some extent. Chondroitin sulfate C is isolated from various connective tissues, including tissue cultures of fibroblasts from bone and from human and rat skin. Chondroitin sulfate A was identified only in the aorta of the cow and in cartilaginous tissues, including a chondrosarcoma, and the *ligamentum nuchae* of the ox.[31] Although no definite conclusion could be established by chemical determination alone, the only inference to be drawn from our investigations is that a certain amount of chondroitin sulfate C is probably present in breast stroma. This is less likely the case with chondroitin sulfate A.

The significance of the acid mucopolysaccharides in breast stroma is difficult to interpret. Increasing amounts of these compounds were noted in the intralobular stroma during the evolutional phase of the menstrual cycle; the reverse was observed in the involutional phase. The increase in acid mucopolysaccharidies was coincident with an increase in stromal cellularity. This portion of the breast is generally

considered to be responsive to estrogen stimulation; in fact, the increment in demonstrable acid mucopolysaccharide occurs during that phase of the cycle when estrin is known to rise. It is noteworthy that during early menopause there is a gradual decrease in the amount of acid mucopolysaccharides; later these disappear almost completely. There is, thus, good reason to assume that a direct relationship exists between estrogen production and the increased amount of acid mucopolysaccharides detectable in the intralobular breast stroma.

There is every indication that the fibroblasts, at least in part, are the elements from which mucopolysaccharides are derived. They were increased in number at the time stainable acid mucopolysaccharide was increased; other cellular elements, such as mast cells and leukocytes, were not so affected. Grossfeld, Meyer and Godman[32] demonstrated the production of hyaluronate and, to a lesser extent, of chondroitin sulfate C by fibroblasts. Others have proposed that hyaluronic acid and chondroitin sulfate were synthesized in the umbilical cord by fibroblasts, and not by mast cells.[33] Although our interpretation of the cyclic variations in acid mucopolysaccharides and of their derivation is by inference, it is felt that no other interpretation is justified with available knowledge and with the observations on our material.

The evidence further indicates that the large amount of acid mucopolysaccharide demonstrated in fibroadenomas and in fibroadenomatous hyperplasia is probably related to abnormal estrogen stimulation or to an abnormal local stromal response to average estrogen action. That the estrogens are involved in the initiation of fibroadenoma of the breast has been repeatedly proposed, although direct proof is lacking.[25] In similar fashion one may suspect the action of estrogens as a basis for the increase of acid mucopolysaccharides in the stroma of most breast carcinomas. On the other hand, it should be noted that an increase of acid mucopolysaccharide content has been found in the stroma of other tumors,[34-36] including neoplasms in which a relation to estrogen stimulation seems remote. Sylvén[34] observed this phenomenon and the added feature that mast cells were also increased in number in this circumstance. He felt that the acid mucopolysaccharides were derived in part from the mast cells and were chemically related to heparin. In our own studies, although metachromasia and mast cells were readily demonstrated in frozen sections of breast carcinoma stained with toluidine blue, it seemed that the number of mast cells was not increased in proportion to the intensity of metachromasia. Moreover, although they were regularly observed in tissue with significant metachromasia, their distribution varied from one portion of a tumor to another, while the metachromasia was, in gen-

eral, uniformly distributed. Some acid mucopolysaccharides (heparin) may indeed be produced by mast cells, but most of them probably originate from other elements.

In gynecomastia the periductal stroma represented another site in which considerable accumulation of acid mucopolysaccharides occurred. It is difficult to relate this finding to the many possible causes of gynecomastia. It is conceivable, at least in some cases, that the lesion might be related to estrogenic stimulation.

In the various forms of mammary dysplasia, a decrease or complete disappearance of acid mucopolysaccharides was manifest to such a degree that no significant difference was noted between the intralobular and the interlobular stroma. This histochemical expression of mazoplasia has been described as the earliest and least extensive form of mammary dysplasia.[37] It was our impression that the first appreciable alteration was a failure of "active stroma" to exhibit cyclic responses. These observations supported the view that the initial step toward fully developed dysplasia occurred in the stroma, more exactly in the intralobular and periductal regions. If one accepts the hypothesis that the variation in acid mucopolysaccharide content of breast stroma during the menstrual cycle is dependent upon estrogenic stimulation, it would follow that whenever such stimulation became irregular, or intralobular stroma lost its capacity to respond, the stroma would cease to exhibit regular qualitative variations. This could, therefore, initiate the chain of events leading to the familiar pattern of mammary dysplasia.

One may speculate that a large part of the normal function of the breast is related to the behavior of its acid mucopolysaccharides. These may, therefore, be considered essential, although intermediary, factors in mammary function, or the histochemical expressions of such function. The first of these interpretations appears more likely, since much evidence has accumulated in the literature indicating the important functional role played by stromal elements.

The interlobular stroma obviously is a rather inactive component of the breast. The carbohydrate nature of its PAS positive substances, established by the acetylation technique of McManus and Cason,[22] led to the conclusion that it was composed of neutral mucopolysaccharides. Its morphologic and histochemical features tend to remain constant at all ages in normal as well as dysplastic conditions. Its function would, therefore, appear to be mainly supportive for the breast as a whole.

The basement membranes seemed to be independent histochemically

of the adjacent periductal stroma. Despite the fact that the latter revealed accumulation of acid mucopolysaccharides during the menstrual cycle and in pathologic states, the basement membranes exhibited a constant PAS positivity. The function of the basement membranes, too, differs from that of the stroma; it serves mainly in support of the epithelium. In noninfiltrating carcinoma, neoplastic cells remained confined by well defined basement membranes. In more advanced stages the membranes of the affected ducts or acini exhibited tinctorial qualities of acid rather than neutral polysaccharide. They appeared considerably thinner and in many instances disappeared entirely. The loss of basement membrane integrity may be presumed to represent one mechanism by which neoplastic invasion occurred.

SUMMARY

A study of the distribution of acid and neutral mucopolysaccharides in the human breast in normal and pathologic states constitutes the basis for this report.

The acid mucopolysaccharides in the periductal and intralobular regions have been tentatively identified and their relationships to mammary function analyzed.

The interlobular stroma, containing a larger amount of neutral mucopolysaccharide, has essentially a supportive function.

The basement membranes were considered to be independent structures with a specialized supportive role in relation to epithelial elements.

An interpretation of the events occurring in the process of carcinomatous infiltration has been proposed.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

All sections shown here stained with the Ritter-Oleson stain.

FIG. 1. Normal duct of adult female mammary gland. Two arrangements of fibers are seen in the wall: circular and longitudinal. × 400.

FIG. 2. Normal lobule of adult female mammary gland in early evolutional phase. No significant difference is noted as yet between the intralobular and the interlobular stroma. × 400.

FIG. 3. Normal lobule of adult female mammary gland in late evolutional phase. Intracytoplasmic PAS positive granules of secretion are evident, as well as accumulation of secretory material in the lumens. In the stained preparation, the intralobular stroma is blue, and the interlobular stroma is purple. × 400.

FIG. 4. Normal menopausal female mammary gland. No difference exists between the interlobular and the periductal stroma. The basement membranes are well preserved. × 200.

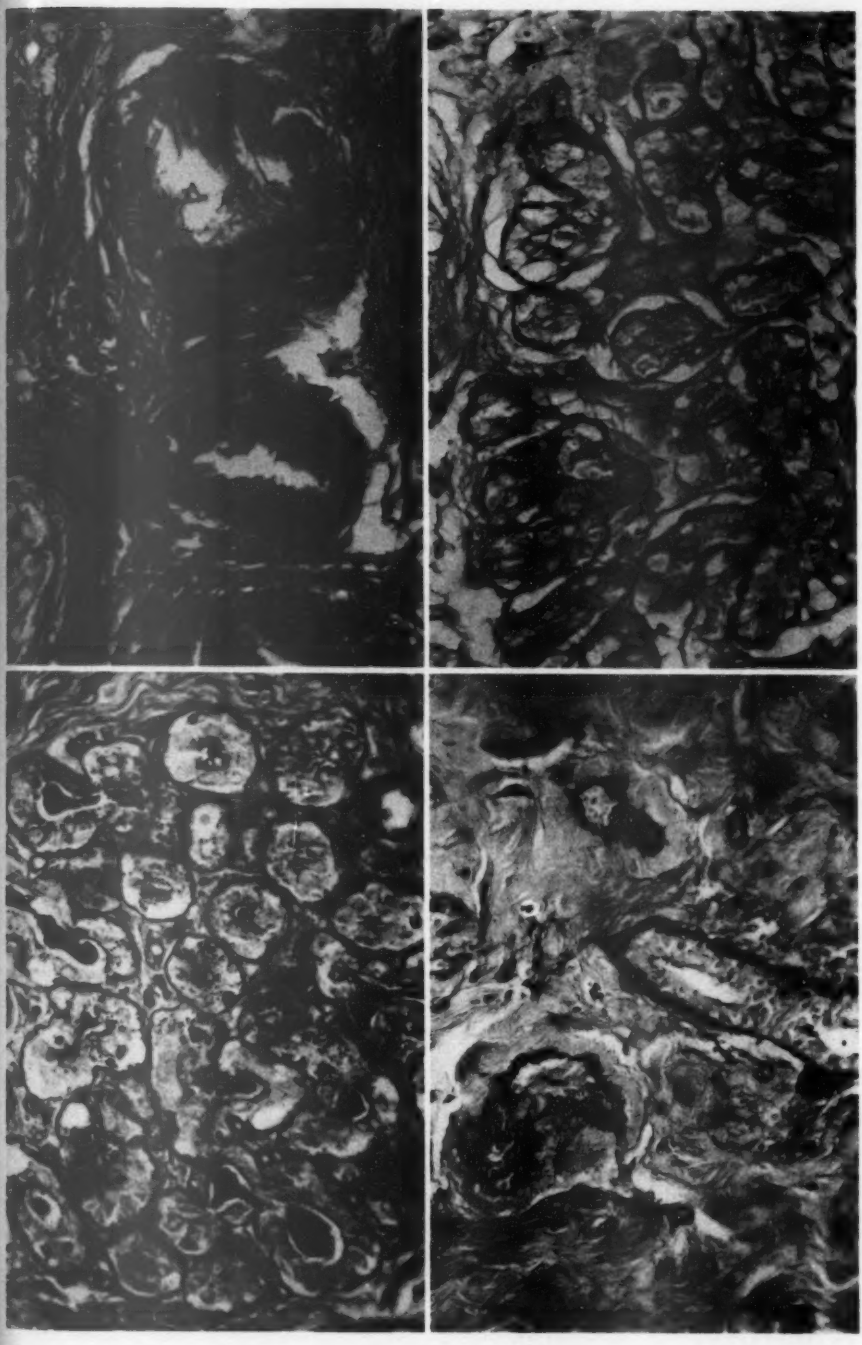


FIG. 5. Apocrine metaplasia in cystic disease of adult female mammary gland. Secretory granules are clearly visible in the cells. × 400.

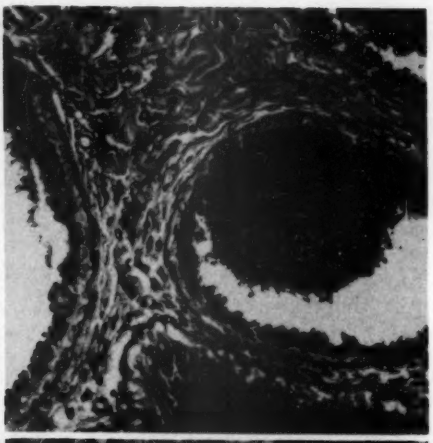
FIG. 6. Cystic disease of adult female mammary gland. The basement membranes are well defined. No significant amount of acid mucopolysaccharides is seen in the stroma in the stained preparation. × 200.

FIG. 7. Sclerosing adenosis in adult female mammary gland. Architectural distortion with persistence of basement membranes is visible. × 400.

FIG. 8. Lobular carcinoma *in situ* of female mammary gland. The nests of neoplastic cells are surrounded by a basement membrane. In the stained preparation, the intralobular stroma is blue in color. × 400.

FIG. 9. Duct cell carcinoma of female mammary gland. A focus of intraductal carcinoma surrounded by a distinct basement membrane is seen in the upper part of the photograph. Below it, the nests of infiltrating carcinoma are devoid of basement membrane. The stroma is stained blue. × 400.

FIG. 10. Gynecomastia in adult male mammary gland. Secreting cells line the duct wall, which is limited by a basement membrane. The periductal stroma, blue in the stained preparation, is looser than the intervening stroma (visible at the upper right corner), which is stained purple. × 200.



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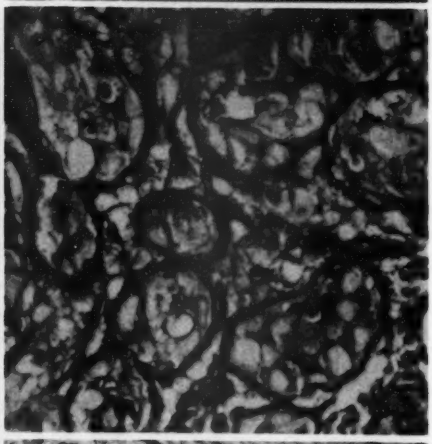

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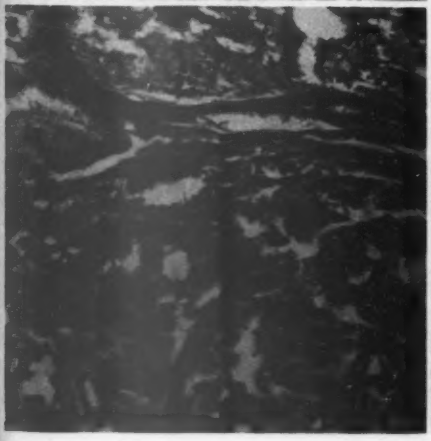

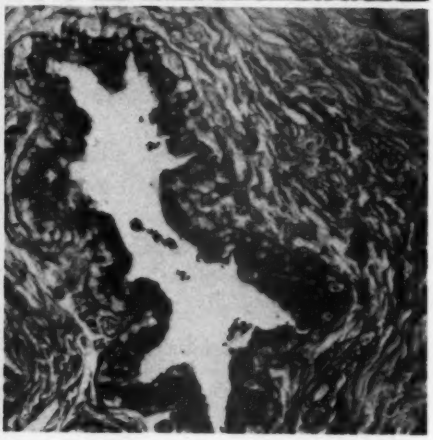

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